The Bee as a Model System for Addiction

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Abstract

The consumption of chemical substances that generate fleeting feelings of pleasure and euphoria, and the progression of dependence to these substances in subsets of individuals, remains a pressing concern for society. Drug addiction carries not only substantial risk to the mental and physical wellbeing of the individual but also carries an enormous social and medical cost, with an annual cost of 15.4 billion pounds a year in the UK alone. There is, therefore, a strong motivation to identify the underlying causes of addiction to aid in identifying future therapeutic strategies.

Historically, mammalian models have been utilised in an attempt to identify the neural substrates involved in the development of addiction. Although these models have proved invaluable in identifying the critical molecular targets of drugs of abuse, much remains unknown about the systems level neural plasticity involved in the development of an addictive state, largely due to the sheer complexity of the mammalian brain.

The insect brain contains many orders of magnitude fewer neurons, but there is remarkable conservation of the molecular mechanisms involved in the encoding of reward. Given this, recently, attention has turned to the use of insects as potential models with which to study addiction. Using a caged behavioural design in the laboratory, I studied the viability of using honeybees and bumblebees as models of addiction for the alkaloids nicotine and caffeine. I found that both honeybees and bumblebees display preferential consumption of these alkaloids and that this preferential behaviour critically depended on both the compound concentration and the schedules of drug administration employed. The study conducted in this thesis is fundamental to being able to use insects to study the neural substrates of addiction. It also sheds further insight into how caffeine and nicotine produced by plants in floral nectar could manipulate bee behaviour and co-evolve to improve plant fitness. Although my work on bee behaviour does not completely validate bees as models for addiction, it lays the groundwork for further studies that may examine maladaptive behaviour in line with the core diagnostic criteria for addiction.

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Table of Contents

List of Figures

List of Tables

List of Extended Data Tables

List of Dose Tables

Abbreviations

A_1, A_{2A}, A_{2B}, and A_3	Adenosine receptor (subscript denotes receptor number)
ACh	Acetylcholine
ANOVA	Analysis of Variance
BI	*Bombus impatiens*
BT	*Bombus terrestris*
CI	Confidence interval
CLO	Clothianidin
CRF	Corticotropin Releasing Factor
CRF	Corticotropin Releasing Factor
DmAdoR	*Drosophila melanogaster* Adenosine Receptor
DPCPX	Dipropylcyclopentylxanthine
DSM-V	Diagnostic and Statistical Manual of Mental Disorders 5
EXT48	Extended Intermittent 48
GABA	Gamma-Aminobutyric Acid
GLM	General Linear Model
GPCR	G Protein Coupled Receptors
GzLM	Generalised Linear Model
HB	Honeybee
HOV	Homogeneity of Variance
I12	Intermittent 12
I48	Intermittent 48
ICD10	World Health Organizations International Classification of Diseases
IMD	Imidacloprid
LC50	Lethal Concentration 50
LSD	Least Significant Differences
mg/kg	Milligram/Kilogram
nAChR	Nicotinic Acetylcholine Receptor
nAChR	Nicotinic Acetylcholine Receptors
NDE	Nicotine Deprivation Effect
nM	Nanomolar

PDE	Phosphodiesterase
PxAdoR	*Plutella xylostella* Adenosine Receptor
RH	Relative Humidity
RM-GLM	Repeated Measures General Linear Model
RNAi	RNA interface
RyR	Ryanodine Receptors
TMX	Thiamethoxam
μM	Micromolar

Chapter 1.0 General Introduction

1.1 Context of the thesis: Honeybees and bumblebees as a model for addiction

Drug addiction is a chronic neurological disorder characterised by compulsive drug use despite serious negative consequences and a persistent vulnerability to relapse. To the individual, drug abuse carries a substantial risk to physical and mental health (El-Guebaly, 2004; Daley, 2013). To society, drug abuse carries an enormous social and medical cost, accounting for 12 % of deaths worldwide (World Health Organisation, 2018) and an annual cost of 15.4 billion a year in the UK alone (Welch et al. 2017). Therefore, there is a strong motivation to identify the underlying causes of addiction to aid in identifying future therapeutic strategies.

Mammalian models of substance abuse have proved invaluable in identifying the neural substrates and pathophysiology involved in drug addiction, yet, our understanding of how drugs of abuse lead to addiction remains incomplete (Huang et al. 2018). Although mammalian models undoubtedly have greater relevance to the human condition, recently attention has turned to the use of insect models (Wolf and Heberlein, 2003; Kaun et al. 2012; Søvik and Barron, 2013; Landayan and Wolf, 2015; Ryvkin et al. 2018; Lowenstein and Velazquez-Ulloa, 2018), primarily due to the insects simpler nervous system, and the identification that the neuronal substrates involved in mediating addiction-like behaviour are conserved across phyla (Waddell, 2013; Scalpen and Kaun, 2016). Indeed, addictive-like behaviour has been observed for a number of addictive substances in honeybees (Barron et al. 2009; Søvik et al. 2014) *Drosophila* (Scholz et al. 2000; Abarca et al. 2002; Devineni and Heberlein, 2009; Lowenstein and Velazquez-Ulloa, 2018), ants (Entler et al. 2016), and crickets (Zabala et al. 1991).

Addiction is considered to be a neuropathological disorder that is both defined and diagnosed by the observation of aberrant behaviour (Volkow and Li, 2004; American Psychiatric Association, 2013), and that arises due to neuroplastic changes in the brain that undermine executive control (Hester et al. 2010). Therefore, it is apparent that any suitable invertebrate model of addiction will require a behavioural repertoire that is sufficiently complex to study the behavioural deficits that arise in the addict, and will be readily amenable to the study of plastic changes that occur during the course of addiction.

Honeybees and bumblebees display arguably the richest behavioural repertoire of any insect species (Scheiner et al. 2013), exhibiting several cognitive capabilities previously only associated with 'higher' vertebrates (Chittka and Niven, 2009). Furthermore, these insects are readily amenable to electrophysiological recordings (Strube-Bloss and Rössler, 2018), despite having a brain ~1 mm^3 containing only ~950,000 neurons (Menzel and Giurfa, 2001). In addition, recent advances in ribonucleic acid interface (RNAi) and clustered regularly interspaced short palindromic repeat (CRISPR) technology in honeybees (Antonio et al. 2008; Wang et al. 2013; Kohno et al. 2016; Costa et al. 2016), indicates the feasibility of genetic manipulation studies in these insects, further validating their use in the study of complex neuropathological diseases. Indeed, in honeybees alone, addictive-like behaviour such as preferential consumption of addictive substances, tolerance, and withdrawal have been observed in response to cocaine (Barron et al. 2009; Sövik et al. 2013) and ethanol (Mustard et al. 2019).

Nicotine and caffeine are drugs that are recreationally used in the general population (Reissig et al. 2009; Prochaska and Benowitz, 2016). Interestingly, these drugs are also known to be found within the nectar and pollen of plants (Detzel and Wink, 1993; Kretschmar and Baumann, 1999; Wright et al. 2013) and have been shown to modulate bee behaviour such as increasing foraging fidelity (Singaravelin et al. 2005; Thompson et al. 2005; Barrachi et al. 2017a), the perception of reward quality (Couvillon et al. 2015), and the memory of the reward (Wright et al. 2013), indicating that these plant compounds may function in an addictive-like manner in these insects. If this is the case, then bees may serve as a viable model with which to study nicotine and caffeine addiction. This review will address the viability of using insects to study nicotine and caffeine addiction. Specific focus will be given to the behavioural paradigms that have been used to generate preferential self-administration of these compounds in other animal models.

1.2 The ecological function of plant secondary metabolites

Plants are under selective pressure to increase the fidelity of animal pollinators whilst deterring the presence of antagonists, such as herbivores (Nielson et al. 2013; Stevenson, 2019). For the former, plant adaptations such as flower morphology, colour, and scent, are well documented (Wright et al. 2009; Krishna and Keasar, 2018). For the latter, plants have evolved mechanical and chemical means to deter herbivorous insects

(Lucas et al. 2000; Agrawal and Weber, 2015). Secondary metabolites are one such class of deterrent chemicals, and their accumulation in the leaves, flowers, and seeds of plants repel insect herbivores due to their bitter taste and toxicity (Nathanson, 1984; Steppuhn et al. 2004; Kessler et al. 2008; Huang et al. 2016).

Secondary metabolites are classified based on their chemical structure, function, and biosynthesis (Thirumurugan et al. 2018). Alkaloids are one class of secondary metabolites and include nicotine, caffeine, and cocaine, amongst others (Aniszewski, 2007). Alongside their presence in the leaves and seeds of the plant (Aniszewski, 2007), alkaloids are also found in much lower concentrations in floral nectar and pollen (Adler and Irwin, 2000). Given that the floral rewards of certain plant species contain compounds that are known to function as addictive substances in mammals, recent studies have focussed on establishing whether these compounds may also exert addictive properties on insect pollinators, and therefore, whether their presence is capable of mediating plant-pollinator interactions.

Perhaps the most well-studied of these alkaloids in bees is caffeine, and extensive evidence exists to suggest that this alkaloid can modulate bee behaviour. For instance, honeybees that forage on caffeinated solutions have been shown to increase both the frequency and likelihood of honeybees performing the waggle dance (Couvillon et al. 2015), a behaviour that is known to correlate with the bees valuation of nectar quality (Von Frisch, 1967; Seeley, 1991), indicating that bees find caffeinated nectar more rewarding than nectar that is absent for the compound. This behaviour results in additional recruitment of foragers to feeders that contain the alkaloid (Singaravelan et al. 2005; Couvillon et al. 2015), leading to greater pollination transfer in artificial flowers (Thompson et al. 2015). Furthermore, bees trained to forage from a feeder containing caffeinated food will continue to return to that feeder even when the food source has been removed (Couvillon et al. 2015). Finally, when honeybees consume caffeinated sucrose solutions during olfactory conditioning, they are three times more likely to remember an odour associated with a reward than bees that have been fed sucrose alone (Wright et al. 2013). An effect that arises due to the pharmacological actions of this alkaloid on the bee's brain (Wright et al. 2013). In an ecological context, this suggests that bees that forage on flowers that have caffeine

within their nectar are more likely to remember the odour associated with that plant than plants that are absent for the compound.

Similar results have been obtained for other alkaloids, where cocaine administration has been shown to increase the frequency of the waggle dance (Barron et al. 2009) and decreases the rate of memory extinction in the proboscis extension response assay in honeybees (Søvik et al. 2018). In addition, Barrachi et al. (2017) identified that even though bumblebees initially avoid high concentrations of nicotine presented in artificial flowers, in a subsequent two-choice test for nicotine-containing flowers, and flowers containing sugar solution alone, they were more likely to remain faithful to flowers that contained nicotine. This was true even when these flowers became the sub-optimal choice, achieved by replacing the nicotine-sucrose solution with water (Barrachi et al. 2017a). Collectively, this suggests that low concentrations of alkaloids may serve as a form of floral deception, manipulating the bee's behaviour to increase pollination services.

Honeybees and bumblebees are both generalist foragers (Geslin et al. 2017), which rely on pollen from a wide diversity of plants to meet their nutritional needs (Donkersley et al. 2017; Lau et al. 2019), and forcing honeybees to forage from a single nutritional source is detrimental to colony growth and fitness (Bonoan et al. 2019). Therefore, not only may alkaloids be capable of manipulating bee behaviour, but this deception may potentially come at the cost of the colony's health. Given that the consumption of alkaloids appears to result in maladaptive behaviour in the bee, and addiction is diagnosed based on the presence of maladaptive behaviours (see later), this suggests that alkaloids may function as addictive compounds in these insects.

1.3 The conserved function of dopamine and its role in addiction

The ability to learn and accurately encode associations between actions and the value of their outcomes is critical to the survival of all animal species. Learning to approach natural rewards, such as food, water, and sex, and avoid negative stimuli such as interactions with predators, or toxic food, allows the animal to secure the necessary elements and relative safety required to ensure the propagation of the species. Although multiple neurotransmitters (Arias-Carrión et al. 2014) and brain regions (Berridge and Kringelbach, 2015) are known to play a part in mediating rewarding and aversive

reinforcement behaviour, dopamine has been shown to be the critical neurotransmitter in encoding valence in mammals (Schultz, 2013), insects (Burke et al. 2012; Waddell, 2013; Lowenstein and Velazquez-Ulloa, 2018), molluscs (Brembs et al. 2002; Reyes et al. 2005; Lorenzetti et al. 2011; Kemenes et al. 2011; Bedecarrats et al. 2013*)*, and worms (Tanimoto et al. 2016; Engleman et al. 2016; Han et al. 2017).

Dopamine is a catecholamine neurotransmitter that is involved in the encoding of motivated behaviour (Berridge and Robinson, 1998), reward prediction error (Schultz, 2016), the modulation of long-term memory (Pignatelli and Bonci, 2015; Waddell, 2013), and in gating appropriate motor outputs in response to both rewarding and aversive stimuli (Freeze et al. 2013). In both insects and mammals, dopamine is known to be released in response to a range of natural rewards, including food (Wang et al. 2011; Waddell, 2013), water (Young et al. 1992; Shyu et al. 2017), sex (Melis and Argiolas, 1995), and social interaction (Manduca et al. 2016; Kim et al. 2018). This release of dopamine encodes a strong positive valence within the reward pathways of the brain and increases the motivation to obtain the reward in the future by mediating the formation of long-term associative memory between the reward and the reward cue (Arias-Carrión et al. 2010; Waddell, 2013).

That dopamine plays a critical role in the development of addiction in mammals is observed by the fact that all known addictive drugs serve to increase extracellular levels of dopamine either directly or indirectly in key brain regions involved in encoding reward-related behaviour (Peña et al. 2016). As such, these addictive compounds are believed to usurp the natural reward circuity, encoding a strong positive valence for the drug and markedly increasing motivated behaviour to obtain the drug in the future (Berridge and Kringelbach, 2008).

At present, the role of dopamine in encoding reward in insects is largely limited to studies involving *Drosophila* due to their amenability to genetic manipulation techniques, which allows for the comprehensive study of insect reward pathways (Owald and Waddell, 2015; Tedjakumala et al. 2017). However, a number of lines of evidence suggest that, similar to other invertebrates, dopamine is the critical neurotransmitter involved in reward encoding in the bee. For example, McNeill et al. (2016) identified that dopaminergic

neurons are preferentially activated in the honeybee brain not only in response to appetitive stimulus (sucrose consumption) but also in response to reward value (high versus low sucrose concentrations). In addition, dopamine receptors and genes involved in dopamine metabolism are known to be upregulated during a range of reward-related behaviours in the honeybee (Naeger and Robinson, 2016; Singh et al. 2018). Finally, Lagisz et al. (2016) identified that distinct polymorphic sequences within subsets of honeybee dopamine receptor subunits were significantly correlated with appetitive learning scores. This suggests that, similar to other invertebrates, dopamine is responsible for encoding elements of reward in the bee. Although no studies to date have assessed the role that dopamine plays in mediating preferential consumption of addictive substances in bees, substantial evidence has arisen from studies in *Drosophila* to suggest that dopamine is critical in mediating addictive-like behaviour to a range of drugs, such as ethanol, cocaine, and amphetamines (reviewed extensively in Søvik and Barron, 2013; Landayan and Wolf, 2015; Ryvkin et al. 2018; Lowenstein and Velazquez-Ulloa, 2018).

1.4. Locomotion behaviour and the study of addiction in mammals and insects

Although not studied directly in this thesis, it is critical to the understanding of the literature that the reader is aware of the use of locomotion studies in assessing drug abuse liability due to the popularity of these studies in assessing addiction. In mammals, all known addictive substances are known to increase and decrease locomotor behaviour upon acute challenge in a biphasic concentration-dependent manner (Calabrese, 2008). The psychomotor stimulant theory of addiction originally proposed by Wise and Bozarth (1987) posits that increased forward horizontal locomotive behaviour following psychostimulant drug administration functions as a reliable biomarker of the addictive nature of the compound. This arises from the fact that addictive compounds function to increase dopamine efflux within the reward centres of the mammalian brain (the nucleus accumbens), and neurons within the reward centres directly modulate locomotor behaviour. Therefore, the theory posits that dopamine mediates both the locomotor and positive reinforcing properties of addictive drugs, and activation of locomotor behaviour can be used to infer the addictive properties of the drug (Reviewed in Vezina et al. 2007; Calabrese, 2008). Indeed, invertebrate addiction literature has similarly centred around identifying increased locomotion upon acute challenge with addictive substances, and biphasic locomotor responses have been observed in *Drosophila* in response to cocaine

(McClung and Hirsh, 1998; Bainton et al. 2000; Filošević et al. 2018), nicotine (Bainton et al. 2000; Hou et al. 2004; Sanchez-Díaz et al. 2015; Zhang et al. 2016) ethanol (Bainton et al. 2000; King et al. 2011), and amphetamines (Pizzo et al. 2013). Similar to mammalian models, this change in locomotor response has been shown to be under the control of dopamine, as the locomotor response to these drugs are attenuated following genetic perturbation of dopamine receptors, or the dopamine transporter, as well as pharmacological reduction of dopamine (Bainton et al. 2000; Li et al. 2000; King et al. 2011; Pizzo et al. 2013; Andretic et al. 2005; Zhang et al. 2016). This biphasic locomotor response has also been observed in honeybees in response to acute challenge with heroin (Fu et al. 2013; Hassanpour-Ezatti, 2015) and ethanol (Maze et al. 2006). Indeed, suppression of dopaminergic neurons in *Drosophila* decreases locomotor behaviour (Fuenzalida-Uribe and Campusano, 2018). Thus, it appears that addictive compounds function to modulate locomotion in a dopamine-dependent fashion in insects, in line with the locomotor changes that occur in mammalian models of addiction.

1.5. Diagnosing addiction and the study of addiction in mammalian and insect models

Addiction is characterised as the transition from occasional volitional drug use to compulsive drug seeking resulting from the development of dependence. The terms tolerance and dependence are often incorrectly used interchangeably, tolerance specifically refers to a diminished response to the drug following its repeated use (Pietrzykowski and Treistman, 2008), whereas dependence occurs after tolerance has manifested and is defined as a physical need for the drug, such that abstinence from the drug leads to symptoms of withdrawal (D'Souza and Markou, 2011). Throughout the process of addiction, the individual also displays a range of maladaptive behaviours, and these behaviours, alongside the physical symptoms of tolerance and dependence, form the basis of the clinical criteria used to diagnose an individual as an addict.

The Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 2013) and the World Health Organizations International Classification of Diseases (ICD10) serve as the two main diagnostic criteria in clinically defining substance abuse in humans (Hasin et al. 2013; Søvik et al. 2013). They specify a range of psychological and physiological symptoms that must exist concurrently in order to characterise an individual as displaying addictive behaviour (Table 1.5). Psychological

Table 1.5 | The similarities and differences in diagnostic criteria for drug addiction as detailed by the DSM-V and the ICD10. Table adapted from Søvik and Barron. (2013).

Name	DSM-V substance dependence	ICD10 dependence syndrome	Differences
Criteria	three or more of the symptoms listed below occurring at any time during a year	three or more of the symptoms listed below occurring together for at least 1 month or repeatedly for shorter periods during a year	
Symptoms			
Physiological symptoms			
Tolerance	√	√	
Withdrawal	√	√	
Psychological symptoms			
Lack of control	√	√	split into two distinct symptoms in the DSM, one focusing on desire and the other on behaviour
Time usage	√	√	split into two distinct symptoms in the DSM, one focusing on time spent and the other on giving up other activities
Continued use despite harm	√	√	knowledge of harm is not necessary in the ICD
Compulsion	X	√	the DSM does not consider compulsion a symptom

√ refers to criteria present in the diagnostic manual. X refers to criteria that are absent.

symptoms include sustained drug use despite adverse consequences, lack of control, an extensive amount of time devoted to drug acquisition, and compulsion, whereas physiological symptoms include tolerance and withdrawal symptoms.

Although tolerance and dependence often develop in synchrony, it is important to note that they represent distinct aspects of addiction. For instance, tolerance may develop to the negative aspects of a drug, such as the drug's ability to cause nausea, but the user may exhibit no symptoms of dependence on the drug. In addition, drug dependence itself is distinct from addiction. A drug user may become physically dependent on a drug such that cessation leads to withdrawal symptoms, but aside from the negative repercussions of withdrawal, displays no other signs of addiction such as continued drug use despite harm, compulsion, etc. A classic example of such drugs are antidepressants which may generate symptoms of withdrawal but do not typically fit the DSM-V or ICD-10 criteria to be characterised as addictive *per se* (Evans and Sullivan, 2014). Indeed, tolerance and dependence, either alone or in combination, are neither necessary or sufficient to classify a human subject as an addict under the current DSM-V and ICD10 criteria, and psychological symptoms must exist concurrently in order to be diagnosed as an addict. Thus, it is critical in mammalian, as well as insect models of addiction, to ascertain that sufficient addiction-like criteria are met to classify the animal as exhibiting drug addiction.

Both mammalian (Spanagel, 2017) and insect models (Lowenstein and Velazquez-Ulloa, 2018) of drug addiction have focused on behavioural techniques and drug-administration schedules capable of generating each of these symptoms. For instance, both rodents (Wolffgramm and Heyne, 1995) and *Drosophila* (Kaun et al. 2012) have been shown to overcome electric shocks to gain access to ethanol, or cues associated with ethanol, thereby fulfilling the criteria of 'continued use despite harm'. Similarly, symptoms of withdrawal are identified by exposing the animal to the drug for extended periods and then forcibly imposing abstinence to identify signs of distress or impaired cognitive functions. For instance, both mammals (Dalley et al. 2005) and honeybees (Barron et al. 2009; Fu et al. 2013) exhibit cognitive defects following withdrawal from cocaine or morphine, as exhibited by decreased response accuracy in the 5-choice serial reaction time test and a decline in learning performance in olfactory conditioning of the proboscis extension reflex, respectively. Finally, tolerance has been observed in response to cocaine in both mammals (Marusich et al. 2008) and honeybees (Søvik et al. 2013) and is observed as an attenuation of the locomotor effects of the drug following repeated exposure to cocaine. Following identification of each specific symptom of addiction, analysis of the relevant changes in the reward system at both the systems and molecular level can be conducted to identify the specific neuroadaptations that occur during the development of addiction.

1.6. The theoretical framework for addiction

Addiction has been conceptualised as a cycle of both positive and negative reinforcement (Fig. 1.6) (Koob and Volkow, 2016). In humans, in the early stages of addiction, drug use is believed to be initiated and maintained primarily due to the hedonic effects of the drug, e.g. euphoria (Wise and Koob, 2014; Berridge and Kringelbach, 2015; Koob and Volkow, 2016). During this initial stage, the elevated dopamine release following drug administration functions as a strong positive reinforcer, thereby facilitating repeated drug self-administration in the future (Volkow et al. 2004; Wise and Koob, 2014). It is important to note that at present, we lack the means to reliably infer affective states in animals (i.e. euphoria). Although superficial evidence exists to suggest that insects experience positive affective states (Perry and Baciadonna, 2011; Barrachi et al. 2017b), it is questionable whether insects may experience euphoria or are capable of acting hedonistically. However, the situation is identical in rodent models of addiction, and there

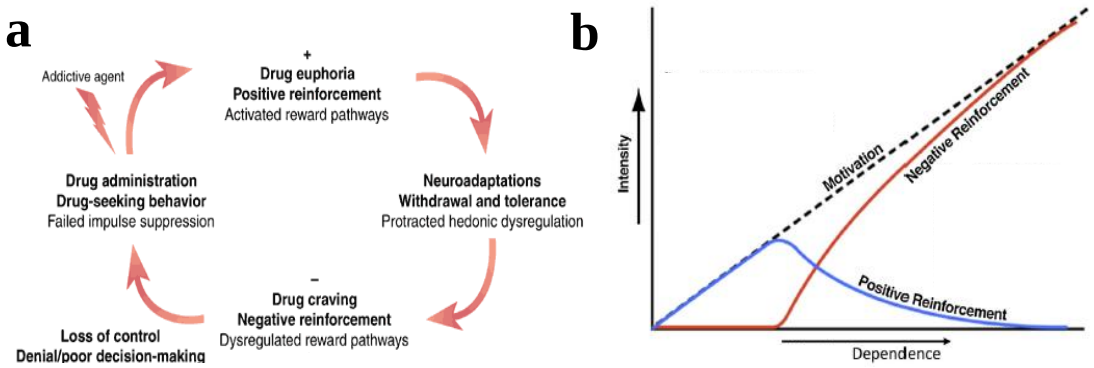

Figure 1.6 | The theoretical framework for the development of addiction. (a) The cycle of addiction: Positive reinforcement, such as the euphoria that drugs produce and the resulting increase in dopamine release in the reward centres of the brain, instil the repeated seeking of the drug in the initial stages. Following repeated drug use, neuroadaptations occur within the central nervous system in a bid to maintain homeostasis. This renders the user susceptible to symptoms of withdrawal, and negative reinforcement occurs in an attempt to overcome the aversive symptoms encountered during drug abstinence. The neuroadaptations that occur in the brain also function to impair executive control, coupled with symptoms of withdrawal; this results in a period of enhanced drug-seeking, increasing the likelihood of repeat drug use. **(b)** Collectively these processes result in a motivational shift over time, with positive reinforcement dominating in the early stages of drug use and negative reinforcement spurring continual drug use following the development of the addictive state.

is no concrete evidence for the subjective components of emotion in these animals (Perry and Baciadonna, 2011). Instead, euphoria is only assumed to be present in rodents based on the animal's intoxication (Jirkof et al. 2019). Therefore, animal models of addiction focus primarily on whether dopamine levels are elevated following drug administration and whether there is behavioural evidence of the drug functioning as a positive reinforcer (e.g. preferential self-administration of the drug).

Following the transition to more frequent drug self-administration, tolerance and dependence begin to develop (Koob and Le Moal, 2008). Tolerance arises not only to any adverse effects that the drug may generate (e.g. nausea) but also to the subjective positive effects of the drug (e.g. euphoria), resulting in a marked increase in the drug dose required to achieve the desired effect, leading to escalated drug use (Edwards and Koob, 2013). Whereas dependence renders the user susceptible to symptoms of withdrawal, resulting in pronounced negative reinforcement behaviour as the user seeks to avoid withdrawal syndrome (Edwards and Koob, 2013).

It is thought that tolerance and dependence arise as a means to maintain neural homeostasis of reward circuitry from the continual demand that addictive substances place on the system (Koob and Le Moal, 2008). The mechanisms underlying the development of tolerance and dependence are highly specific and vary depending on the drug in question. Tolerance commonly arises due to specific changes in drug target receptor activity, such as altered receptor expression or ligand affinity (Quarta et al. 2004; Renda and Nashmi, 2014), whereas dependence is generated not only through changes in drug target receptors but may also occur due to non-target changes, such as the recruitment and sensitisation of stress-response pathways that contribute to symptoms of withdrawal (Wise and Koob, 2014; Koob and Volkow, 2016), and function to interfere with baseline levels of dopaminergic transmission following drug cessation (Parsons et al. 1991; Weiss et al. 1992; Shen et al. 2003; Zhang et al. 2012; Grieder et al. 2012). Over time as drug use increases and tolerance and dependence are established, the motivation to continue drug self-administration begins to shift away from positive reinforcement, and negative reinforcement dominates (Fig. 1.6a & b).

1.7. Nicotine addiction

Nicotine is an alkaloid found in the leaves and flowers of *Nicotiana* spp. (Singaravelan et al. 2005; Adler et al. 2006; Tadmor-Melamed et al., 2004; Kessler et al., 2010), and is the primary psychoactive agent within cigarette smoke (Benowitz, 2010). Low doses of nicotine in drug naïve mammals serve to improve cognitive performance (Wignall and de Wit, 2011), memory consolidation (Beer, 2016), and induce mild euphoria (Agué, 1973; Benowitz, 1988). In comparison high doses are anxiogenic (Anderson and Bruznell, 2015) and may cause nausea and vomiting (Callahan-Lyon, 2014). According to a recent report from the World Health Organisation (WHO, 2018), there are an estimated 1.1 billion smokers globally, of which 50 % will die as a direct result of their tobacco addiction. Smoking alone is believed to be the single leading cause of preventable disease and mortality in both the UK and USA (Thun et al. 2012), and globally, the cost of nicotine addiction leads to an estimated half a trillion dollars a year in economic damage (Ekpu and Brown, 2015).

Remarkably, despite the damage that nicotine causes to both the individual and to society, nicotine is the least studied addictive agent in experimental animal models of

addiction (cocaine studies account for ~40 % of publications, and nicotine < 10%) (Ahmed, 2010), and the molecular and neurological alterations that result in the development of nicotine addiction are far from clear (Benowitz, 2010; Brunzell et al. 2015). Given the cost of smoking to both the individual and to society, it is imperative that we identify the underlying causes leading to nicotine addiction to direct future cessation strategies.

1.7.1 Nicotine functions as an agonist of nicotinic acetylcholine receptors

There are two main classes of acetylcholine (ACh) receptors in insects and mammals: the metabotropic muscarinic acetylcholine receptors (Collin et al. 2013; Haga, 2013) and the ionotropic nicotinic acetylcholine receptors (nAChR) (Albuquerque et al. 2009). Nicotine functions as a highly specific agonist of only the nAChRs, and as such only nAChRs are believed to be involved in the development of nicotine addiction (Dani, 2015). Binding of nicotine to nAChRs in the mammalian brain results in a release of dopamine in the reward centres of the brain (Marshall et al. 1997; Balfour and Benwell, 1992; Balfour, 2015).

nAChRs are heteropentamer complexes composed of five subunits (Albuquerque et al. 2009; Fasoli and Gotti, 2015). Mammals have 16 nAChR subunit types; however, only 11 of these subtypes are present within the CNS: nine α subunits (α2-α7, & α9-α10) and three β subunits (β2-4) (Brunzell et al. 2015), of which only a subset of these subunits are expressed within the reward centres of the brain (Perry et al. 2002; Azam et al. 2002; Grady et al. 2007; Yang et al. 2009). nAChRs are either homomeric (i.e. composed of all one receptor subtype) or heteromeric (i.e. composed of different receptor subtypes) (Fasoli and Gotti, 2015). To further complicate the function of nAChRs, heteropentamers vary in their stoichiometry and in the presence or absence of an accessory subunit, which can dramatically change the functional properties (e.g. Ca^{2+} permeability) and nicotine binding affinities of the subunit (Fasoli and Gotti, 2015). As such, mammalian nAChRs have the potential to form thousands of pharmacologically unique combinations, and at present, the exact function and stoichiometry of nAChRs involved in nicotine addiction in mammals are still unknown (Brunzell et al. 2015).

nAChRs can be in either an open, resting, or closed state depending on the presence of agonists (Dani et al. 2000; Quick and Lester, 2002; Wooltorton et al. 2003; Giniatullin

et al. 2005). Acute application of ACh stabilises the receptor into the open formation for several milliseconds before allowing the receptor to return to the resting state (Dani et al. 2000; Quick and Lester, 2002; Wooltorton et al. 2003; Giniatullin et al. 2005). Unlike ACh, which is hydrolysed by acetylcholinesterase resulting in termination of transmission, nicotine does not undergo degradation, and under prolonged nicotine exposure the receptors desensitise such that they are unresponsive to further agonist stimulation (Dani et al. 2000; Quick and Lester, 2002; Wooltorton et al. 2003; Giniatullin et al. 2005). Receptors that contain the β2 subunit have a high affinity for nicotine, do not bind to α-bungarotoxin, and tend to desensitise slowly (Fenster et al. 1997; Cordero-Erausquin et al. 2000; Papke et al. 2001; Dani, 2015). Conversely, receptors containing the α7 subtype have a lower affinity for nicotine, bind to α-bungarotoxin, and desensitise rapidly (Cordero-Erausquin et al. 2000; Papke et al. 2001; Dani, 2015).

Honeybees and bumblebees have 11 nAChRs (Jones et al. 2006; Sadd et al. 2015), whereas *Drosophila* have only 10 (Jones and Sattelle, 2010). Similar numbers of nAChRs are reported in other insect species (Jones and Sattelle, 2010). Honeybee nAChRs share between 34-84 % sequence identity with *Drosophila* nAChRs (Jones et al. 2006), and individual honeybee nAChRs share similar sequence identity to human nAChRs. For instance, although the honeybee α8 shares 47 % identity with the human α2 receptor, it also shares between 37-47 % identity with the human α3-α9 nAChRs (Thany et al. 2003; Jones et al. 2006). Given such close identity with numerous human nAChRs makes inferring their functionality (e.g. likely nicotine affinity or ion permeability) from orthology alone unreliable (Pearson, 2013). In addition, alternative splicing and A-to-I pre-mRNA editing have both been observed in *Drosophila* and honeybee nAChRs (Jones et al. 2006; Sattelle et al. 2005), indicating that the number of final insect nAChRs is likely to be highly expanded due to these post-transcriptional modifications (Sattelle et al. 2005; Jones and Sattelle, 2010). Finally, co-immunoprecipitation studies have indicated that insect nAChRs, similar to mammalian nAChRs, are likely to form both homomers and heteropentamers (Marshall et al. 1990; Jones and Sattelle, 2010). However, as in mammals, the exact subunits within each heteropentamer are unknown (Jones and Sattelle, 2010). Therefore, the potential exists for insect nAChRs to form thousands of pharmacologically unique combinations of receptors (Jones and Sattelle, 2010), similar to mammals.

Honeybee nAChRs have been shown to have both α-bungarotoxin sensitive and insensitive nAChR subtypes (Goldberg et al. 1999; Barbara et al. 2008; Dupuis et al. 2011) that become desensitised following prolonged exposure to nicotine (Goldberg et al. 1999; Barbara et al. 2008; Dupuis et al. 2011). In addition, binding of nicotine to nAChRs has been shown to result in dopamine release in the brain of the *Drosophila* in a concentration-dependent manner (Pyakurel et al. 2018; Shin and Venton, 2018). Although bumblebee nAChR subtypes have yet to be assessed for their α-bungarotoxin sensitivity and desensitisation in response to nicotine, their high orthology to honeybee nAChRs (~99% sequence identity) (Sadd et al. 2015) suggests that the bumblebees nAChR subunit responses to nicotine will likely follow that seen in the honeybee.

In order to utilise insects as models of addiction, there needs to be at least reasonable semblance and conservation of drug target receptors. Although at present insect nAChRs are understudied in comparison to their mammalian counterparts, it is clear that both insects and mammals have a potentially vast repertoire of nAChRs, both of which form homomers and heteropentamers, can be defined pharmacologically based on their sensitivity to α-bungarotoxin, are desensitised in the presence of nicotine, and function to release dopamine following nicotine administration. This similarity indicates that nAChRs exhibit reasonable conservation across phyla and justifies the further study of insects as models of nicotine addiction.

1.7.2 Modelling the stages of nicotine addiction in animal models: Lessons from rodents

The first stage in establishing whether an animal displays addictive-like behaviour to a substance is to identify whether the animal will self-administer the compound, at what concentration, and under which protocol of administration (Koob and LeMoal, 2006). When the compound dose is low, the concentration is insufficient to activate reward circuitry, and therefore no self-administration occurs. Whereas when the compound dose is high, reward circuitry may be activated; however, the aversive side effects of the compound typically occur, resulting in little or no self-administration (Koob and LeMoal, 2006). This leaves a narrow dose-range in which animals will reliably self-administer a drug, resulting in a biphasic or inverted U-shaped dose-response curve (Fig. 1.7.2.a); or a

J-shaped response curve, depending on the end-point measured (Haney and Spealman, 2008; Koob, 2008; Calabrese, 2008). This biphasic dose-response curve is common to all drugs of abuse, including caffeine, nicotine, cocaine, and ethanol (LeFoll et al. 2007; Calabrese, 2008). In addition, this response curve can shift to the right in animals that are highly dependent on the drug, whereby higher concentrations are required to activate reward circuitry, and the animal is more tolerant of the aversive effects that occur in response to high doses of the compound (Koob and LeMoal, 2006).

Mammalian models of nicotine addiction can, broadly speaking, be separated into four distinct protocols of drug administration: (i) Short access paradigms reflecting early nicotine experimentation, (ii) long access paradigms reflecting the development of tolerance and dependence, (iii) pre-exposure access paradigms which 'fast-tracks' the animal to a nicotine-dependent state, and (iv) intermittent access paradigms which more closely resemble the human condition and can be used to study negative reinforcement behaviour in more detail.

Short access paradigms reflect the very early stages of nicotine addiction, such as when a smoker is using cigarettes only sporadically. In humans, this represents the stage where neither tolerance to the positive effects of the drug has occurred or dependence has developed (Shiffman, 2009; Shiffman and Paty 2006; Goedeker and Tiffany, 2008), and primarily reflects the peak hedonic period of nicotine addiction (Shiffman and Paty, 2006). In rodent models, this stage is typically replicated by allowing rodents very short periods of nicotine access a day (~1-2 h) to intravenously self-administer the drug by pressing a lever for nicotine infusions. These short access protocols do not result in nicotine dependence, and animals do not experience withdrawal (Watkins et al. 1999; Baker et al. 2013; Cohen et al. 2015), indicating that these paradigms solely reflect the rodents' hedonic responses to the drug.

Short access paradigms of administration result in facilitated dopamine release following each period of nicotine administration (Marshall et al. 1997; Benwell and Balfour, 1992; Balfour et al. 1998; Cadoni and Di Chiara, 2000; Di Chiara, 2000; Schoffelmeer et al. 2002; Rahman et al. 2004; Baker et al. 2013; Balfour, 2015). This enhancement of dopamine release functions to explain why smokers are motivated to

increase their nicotine use in the initial stages, as essentially each cigarette encodes a positive valence greater than the last (Baker et al. 2013; Vanderschuren and Pierce, 2010). This increased dopamine release is reflected behaviourally as an increase in locomotor behaviour with each administration of the drug (Schoffelmeer et al. 2002; Tapper et al. 2004; Baker et al. 2013; Balfour, 2015), a response termed 'locomotor' or 'behavioural sensitisation', in line with the psychomotor stimulant theory of addiction (Wise and Bozarth, 1987; Robinson and Berridge, 1993). In addition, although tolerance does not occur to the hedonic effects of nicotine in short access paradigms, tolerance typically develops to the adverse properties of the drug (e.g. malaise) (Stolerman et al. 1974) and is believed to be brought about by nAChR desensitisation (Picciotto et al. 2008). This further allows the animal to increase their use of nicotine unimpeded by nicotine's aversive properties. Graphically this stage of addiction is represented in figure 1.7.2b as an increase in the intensity of positive reinforcement during the early stage of nicotine dependence.

Long access paradigms reflect the next transition stage in the development of addiction, where the smoker is gradually increasing their cigarette use on a daily basis due to the development of tolerance. Tolerance in mammals is mediated by specific upregulation of nAChR subunits (McCallum et al. 2006; Nashmi et al. 2007; Tapper et al. 2007; Renda and Nashmi, 2014; Meyers et al. 2015). At this stage, elements of positive reinforcement may coexist simultaneously with the development of dependence in smokers (Shiffman and Kirchner, 2009; Caraballo et al, 2009); however, it is specifically dependence (i.e. the development of withdrawal) that functions as the critical driver for increased nicotine use (Caraballo et al. 2009). The specific neuroadaptations that lead to dependence in mammals are not currently known, but evidence exists for upregulation of specific nAChR subunits (Buisson and Bertrand, 2002; Govind et al. 2009; Brunzell et al. 2015) and increased expression of the stress hormone, corticotropin releasing factor (CRF) (George et al. 2007; Cohen et al. 2012; Grieder et al. 2014; Cohen et al. 2015). Long access paradigms are essentially identical to short access paradigms, with the exception that the rodents are allowed ~23-24 h nicotine access a day.

Long access paradigms reflect a stage in nicotine addiction where the animal still receives some elements of positive reinforcement from the drug, as evidenced by the ability of nicotine to stimulate dopamine release in reward centres (Marshall et al. 1997; Kenny

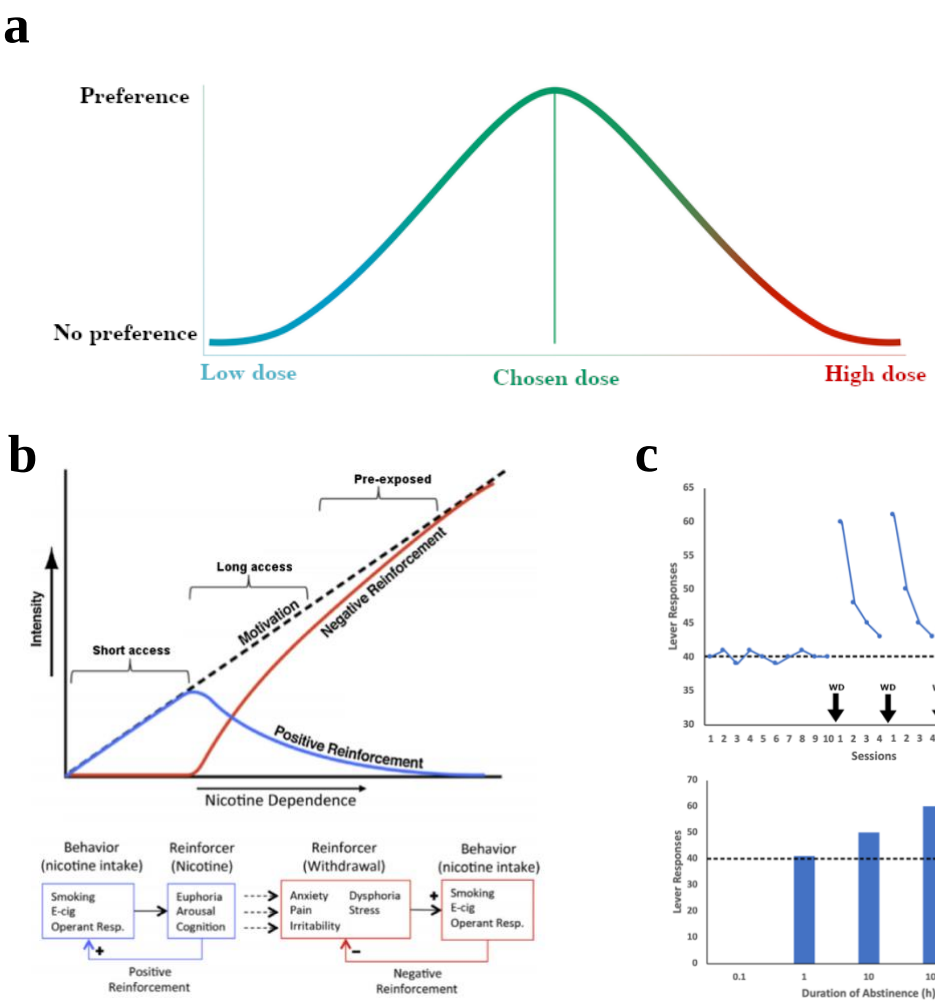

Figure 1.7.2 | Behavioural paradigms used to study nicotine addiction. **(a)** A biphasic dose-response is observed for all known drugs of abuse in mammals. No preference is observed for the drug when concentrations are too low or too high. Preferences are only observed within a narrow dose range. This narrow range represents concentrations that are capable of activating reward circuitry without leading to aversive side effects. **(b)** Short access paradigms reflect the early stwages of addiction where drug use is maintained primarily for the hedonic properties of the drug (i.e. positive reinforcement), and dopamine release is facilitated with each subsequent exposure of the drug, resulting in enhanced positive reinforcement. Long access paradigms bypass the dopamine facilitation stage entirely and represent the more intermediate stages of addiction whereby drug use is maintained due to both positive and negative reinforcement, and typically these protocols result in a robust state of dependence following as little as 1-2 weeks access to the drug. Pre-exposure paradigms bypass both the hedonic and intermediate stages of addiction and instead place the animal into a state of dependence where very little nicotine is administered for hedonic purposes. Bottom: Represents the behaviour and the reinforcer of that behaviour across the development of addiction as it is in the smoker. Figure taken from George and Koob (2017). **(c)** Intermittent paradigms lead to an enhanced period of nicotine self-administration. Top: Rodents that have undergone 10 days of long access (23 h/day) nicotine self-administration (pressing a lever to administer nicotine), followed by 3 days of forced abstinence, display robust "drug loading" once nicotine access is returned. Bottom: The duration of forced abstinence on subsequent drug loading behaviour increases over time (Graphs modified from George et al. 2007).

and Markou, 2006; Grilli et al. 2009); however, typically, this dopamine release is substantially attenuated in comparison to short access protocols (Benwell and Balfour, 1997; Caillé et al. 2009; Kenny and Markou, 2006; Baker et al. 2014). In addition, animals display robust symptoms of withdrawal (O'Dell et al. 2007; Cohen et al. 2012; Cohen et al. 2015; George and Koob, 2017), indicating the transition to a dependent state. Long access paradigms, therefore, closely reflect the transition stage of addiction observed in smokers. The critical difference between short access and long access paradigms is that dopamine release is not facilitated with each period of nicotine self-administration, nor is behavioural sensitisation observed (Benwell and Balfour, 1997; Vezina, 2007; Caillé et al. 2009; Baker et al. 2014; Vanderschuren and Pierce, 2010), a fact likely explained by the specific changes in neuroplasticity that occur during either schedule of administration (Vezina, 2007; Vanderschuren and Pierce, 2010). This indicates that long access protocols are incapable of stimulating the same level of positive reinforcement as short access protocols. Graphically this is represented in figure 1.7.2b as the transition stage whereby negative reinforcement gradually dominates over positive reinforcement.

Pre-exposure protocols are used to 'fast-track' the animal into a dependent state by facilitating neurological changes in advance of self-administration protocols (Damaj et al. 2003; Grabus et al. 2005 Gould et al. 2012). How effective the protocol is, depends on the duration of the pre-exposure period, with longer durations of pre-exposure resulting in both increased symptoms and durations of withdrawal syndrome (Damaj et al. 2003; Skjei and Markou, 2003; Vann et al. 2006). Pre-exposure protocols involve chronically administering nicotine by adding nicotine to the animals only water source (Pietilä et al. 1998; Grabus et al. 2005), and pre-exposure periods of as little as 2-4 days result in a nicotine-dependent state in rodents (Gould et al. 2014; Muelken et al. 2015).

Shorter durations of pre-exposure (~2-14 days) facilitate the animal into a dependent state similar to that observed in long access protocols, with evidence of both positive and negative reinforcement mechanisms present (Benwell and Balfour, 1997; Marshall et al. 1997; Kenny and Markou, 2006; Grilli et al. 2009; Caillé et al. 2009; Baker et al. 2014; Muelken et al. 2015). In contrast, extended pre-exposure paradigms (~3-6 months) result in a blunted dopaminergic response more closely resembling that of heavily

dependent users (Fig 1.7.2b) (Perez et al. 2012; Perez et al. 2013; Exley et al. 2013). Following the development of dependence, the experimenter can then allow the animal to begin nicotine self-administration to study how effective the priming protocol was in establishing addictive-like behaviour. Pre-exposure to nicotine for 10-14 days results in an immediate enhancement of nicotine self-administration in comparison to animals that have not been pre-exposed to the drug (Natividad et al. 2013; Renda and Nashmi, 2014).

Finally, intermittent paradigms introduce single or multiple periods of forced nicotine cessation into the standard long access or pre-exposed methods of nicotine administration by temporarily removing nicotine access. These intermittent paradigms either use relatively short periods of nicotine cessation (e.g. removing nicotine access for ~12 h a day) to more accurately reflect the human condition by mimicking the overnight abstinence smokers experience (George et al. 2010; Gilpin et al. 2014; Baiamonte et al. 2014; Brynildsen et al. 2016), or longer periods (e.g. days to weeks) to allow greater assessment of negative reinforcement behaviour across time (O'Dell and Koob, 2007; George et al. 2007; Nesil et al. 2011; Cohen et al. 2012; Gilpin et al. 2014; Cohen et al. 2015). Animals that have undergone prior long access to nicotine, followed by a forced period of nicotine abstinence exhibit a transient but robust increase in nicotine self-administration (Fig. 1.7.2c, top), that increases with the duration of nicotine abstinence (Fig, 1.7.2c, bottom), a behaviour termed the "nicotine deprivation effect" (NDE). The NDE is thought to represent enhanced negative reinforcement behaviour due to the extended periods of withdrawal (George and Koob, 2017). These intermittent paradigms have been critical in identifying the role that CRF plays in addiction, as abstinence-induced increases in nicotine self-administration behaviour have been shown to be entirely dependent upon increased CRF signalling during nicotine withdrawal (George et al. 2007; Cohen et al. 2012; Grieder et al. 2014; Cohen et al. 2015; Zhao-Shea et al. 2015).

Importantly, intermittent paradigms have been shown to result in an increased motivation to acquire nicotine (Cohen et al. 2012; Cohen et al. 2015) and result in more robust withdrawal symptoms (George et al. 2010; Baiamonte et al. 2014; Brynildsen et al. 2016) in comparison to long access protocols alone. Indeed, the greater the number of withdrawal periods experienced by the animal, the more pronounced the symptoms of withdrawal (Skjei and Markou, 2003; Gilpin et al. 2014), indicating the importance of

introducing intermittent periods into animal models of addiction to truly reflect the human condition. The fact that withdrawal symptoms become more pronounced with each intermittent abstinence period has led to the 'hedonic homeostatic dysregulation' model of nicotine addiction. This model posits that stress response systems, primarily mediated by CRF, become sensitised (i.e. hyperresponsive), each time the animal experiences nicotine intermittently, and it is this sensitised stress response that accounts for the primary withdrawal symptoms following nicotine cessation (Reviewed extensively in Koob and LeMoal, 2006; Koob, 2008; Koob, 2010; George and Koob, 2017).

1.7.3 Modelling the stages of nicotine addiction in animal models: Lessons from insects

To date, the most extensive studies into the effects of nicotine self-administration in invertebrates have been conducted in bees. Note that bee research has focused not only on nicotine but also on the agricultural pesticides neonicotinoids (neo: "a new form of", nicotinoids: "nicotine"), which, similar to nicotine, bind to nAChRs resulting in their desensitisation (Palmer et al. 2013; Moffat et al. 2016), and facilitating dopamine release in insects (Pyakurel et al. 2018). The critical difference between neonicotinoids and nicotine is that neonicotinoids bind to nAChRs with much greater affinity (Yamamoto and Casida, 1999), and therefore substantially lower (nM versus μM) concentrations are required to result in a pharmacological effect.

Studies in both honeybees and bumblebees have identified that both nicotine and neonicotinoids appear to function as a positive reinforcer in these insects, as observed by preferential consumption of these drugs (Summarised in Table 1.5.3). At present, these studies roughly span those observed in mammalian models, with short access (e.g. hours) (Singaravelan et al. 2005; Barrachi et al. 2015; Barrachi et al. 2017a), intermediate length (e.g. a single 24 h period) (Tiedeken et al. 2014; Kessler et al. 2015; Palmer-Young et al. 2017), and long access (10-30 days) (Barrachi et al. 2015; Arce et al. 2018) paradigms all present, and all resulting in preferential consumption of the drug in a concentration and schedule specific manner. Notably, however, these access paradigms are lacking both the pre-exposure and intermittent access schedules. In addition, in comparison to neonicotinoids, preferential consumption of nicotine has not yet been conducted systemically across bee species.

Table 1.5.3 | Choice assays conducted in honeybees or bumblebees for agonists of nAChRs. Imidacloprid (IMD), thiamethoxam (TMX), and clothianidin (CLO). Preferences are listed in green, indifference in black, and aversion in red.

Compound (Species)	Concentration	Method	Citation
Apis mellifera			
Nicotine	**3**, **6**, 12, **15**, **30**, **60**, **120** μM delivered in 0.6 M sucrose	1 h free-flight two-way choice between either nicotine in sucrose or equimolar sucrose.	Singaravelan et al. 2005
TMX, IMD, CLO	TMX: 0.001, **0.01**, **0.1**, **1** μM IMD: 0.001, 0.01, **0.1**, **1** μM CLO: 0.001, 0.01, 0.1, 1 μM All delivered in 0.5 M sucrose.	Caged 24 h two-choice assay between each pesticide at each concentration in sucrose or equimolar sucrose.	Kessler et al. 2015
Bombus terrestris (Bt) / Bombus impatiens (Bi)			
Nicotine (*Bt*)	1, 10, 100, **1000**, 10000 μM All delivered in 0.5 M sucrose.	Caged 24 h two-choice assay between each concentration of nicotine in sucrose or equimolar sucrose.	Tiedeken et al. 2014
TMX, IMD, CLO (*Bt*)	TMX: **0.001**, **0.01**, 0.1, 1 μM IMD: **0.001**, 0.01, 0.1, 1 μM CLO: 0.001, 0.01, 0.1, 1 μM All delivered in 0.5 M sucrose.	Caged 24 h two-choice assay between each pesticide at each concentration in sucrose or equimolar sucrose.	Kessler et al. 2015
Nicotine (*Bt*)	**12** μM delivered in 0.9 M sucrose	Bees infected with *Crithidia bombi.* (i) Bees housed in a petri dish. Continual two-way choice between nicotine or equimolar sucrose. Preference assessed over the length of the bee's life (~ 30 days). (ii) Free-flight experiment. Choice between artificial flowers containing nicotine or flowers containing equimolar sucrose. Preference for nicotine only observed in infected bees.	Barrachi et al. 2015
Nicotine (*Bi*)	12 μM delivered in 0.9 M sucrose	Caged bees infected with *Crithidia bombi.* 24 h two-way choice between nicotine in sucrose or equimolar sucrose.	Palmer-Young et al. 2017
Nicotine (*Bt*)	**6**, 12, **300** μM delivered in 0.9 M sucrose	Free-flight experiment. Choice between artificial flowers containing nicotine in sucrose or flowers containing equimolar sucrose.	Barrachi et al. 2017a
TMX (*Bt*)	**0.007** or **0.038** μM delivered in 0.9 M sucrose	Free-flight experiment. 10 day three-way choice between artificial flowers containing 0.007 μM TMX, 0.038 μM TMX in sucrose, or equimolar sucrose.	Arce et al. 2018

In contrast to the bee, the vast majority of nicotine studies in other insect models have instead relied on inferring the hedonic properties of nicotine through evidence of locomotor sensitisation that model the mammalian short access paradigms, opposed to assessing preferential consumption of the drug. Repeated nicotine exposure increases locomotion in *Drosophila* in a biphasic dose-response manner (Bainton et al. 2000; Hou et al. 2004; Ren et al. 2012; Sanchez-Díaz et al. 2015; Zhang et al. 2016) and results in enhanced locomotion in the honeybee (Michelsen and Braun, 1987), strongly suggestive of facilitated dopaminergic release (Vezina et al. 2007; Baker et al. 2013). Indeed, pharmacological reduction of dopamine levels in the fly was shown to reduce the locomotor-stimulating effects of nicotine, but not basal locomotor behaviour (Bainton et al. 2000). In addition, genetic suppression of dopamine receptor expression within the reward centres of the *Drosophila* brain was shown to be, at least in part, responsible for nicotine's locomotor enhancing effect in this insect (Zhang et al. 2016), strongly suggesting that locomotor sensitisation, and indeed the positive reinforcing effects of nicotine are under the control of dopamine release in the insect. The fact that locomotor sensitisation is present in insects not only strongly suggests that nicotine has positive reinforcing properties mediated by dopamine release but also indicates that the initial stage of nicotine addiction in insects may be functionally similar to that in the mammal, resulting in a hyperresponsive reward system in the early stages of addiction.

Limited reports of nicotine tolerance are present in the invertebrate literature. In the nematode, *Caenorhabditis elegans*, tolerance to nicotine is observed following chronic nicotine administration and is observed as a reduction in the locomotor enhancing effects of the drug, an effect mediated by nAChR upregulation (Feng et al. 2006; Polli et al. 2015). Similar results have been obtained in the flatworm (*Dugesia dorotocephala*) (Rawls et al. 2011). Studies assessing nicotine dependence are similarly limited; however, upregulation of nAChRs has been observed in the honeybee following 3 days chronic exposure to the drug (Christen et al. 2016), indicating that at least some of the underlying molecular adaptions to chronic nicotine are conserved across phyla. In addition, in the flatworm *Planaria*, withdrawal has been observed following cessation of chronic (24 h) treatment with nicotine and is observed as an increase in squirming behaviour (Bach et al. 2016) and reduced motility (Rawls et al. 2011). Alterations in locomotive behaviour have also been

observed in *C. elegans* following cessation from chronic nicotine administration (Feng et al. 2006; Polli et al. 2015).

1.8 Caffeine addiction

Caffeine (1,3,7-trimethylxanthine), a purine alkaloid, is the most widely consumed psychoactive drug in the world, with over 85 % of the world's population consuming caffeinated products daily (DSM-V, 2013). Repeated caffeine consumption is known to lead to signs of dependence and withdrawal upon abrupt cessation, and it is believed that anywhere between 20-80% of regular caffeine consumers exhibit signs of caffeine dependence (Temple, 2009).

Although both the positive (e.g. cognitive enhancing) and negative (e.g. anxiogenic) reinforcing properties of caffeine consumption in humans is well established (Einöther and Giesbrecht, 2013; Meredith et al. 2013), studies assessing whether caffeine alone can establish self-administration in animal models of addiction are both limited and variable (Nehlig, 2018). The DSM-V currently recognises both caffeine intoxication and caffeine withdrawal as clinically diagnosable criteria, whereas caffeine use disorder (i.e. addiction) is not currently recognised (DSM-V, 2013). Despite this, certain DSM-V criteria for addiction, such as 'unsuccessful efforts to cut down or control caffeine use', and 'continued use despite harm' have been observed in a wide number of clinical studies (For recent reviews, see Meredith et al. 2013 and Addicott, 2014). In contrast to the DSM-V, the ICD-10 acknowledges caffeine dependence syndrome as a diagnosable condition (Meredith et al. 2013).

In recent years caffeine has become more common in human food products, including chocolate, non-cola soft drinks, sweets, ice cream, yoghurts, breakfast cereals, beef jerky, bottled water, chewing gum, crisps, and even waffles (Keast and Riddell 2007; Panek et al. 2013; Temple, 2009; Kole and Barnhill, 2013). Of particular concern is the effect of caffeine in adolescents, as the adolescent brain is particularly susceptible to the development of addiction (Winters and Arria, 2011), and symptoms of caffeine dependence and withdrawal have been observed in children as young as 9 years old (Temple, 2009). In addition, the exponential increase in the consumption of energy drinks in adolescents has raised a number of concerns for physical as well as mental health (Reissig et al. 2009; Ishak

et al. 2012; Ibrahim and Iftikha, 2014; Alsunni, 2015; Nowak and Jasionowski, 2015), given the excessive caffeine content that these drinks contain (Reissig et al. 2009).

1.8.1. Caffeine functions as an antagonist of adenosine receptors

Caffeine's main targets in the nervous system are adenosine receptors. Adenosine receptors are 7-transmembrane G-coupled protein receptors that are activated by the endogenous nucleoside, adenosine. Four mammalian subtypes exist; A_1, A_{2A}, A_{2B}, and A_3 (Sheth et al. 2014). Only the A_1 and A_{2A} receptors are present in high densities within the reward centres of the mammalian brain (Dixon et al. 1996; Rosin et al. 2003; Jacobson and Gao, 2006). Caffeine functions as a non-selective antagonist of both A_1 and A_{2A} receptors (Ribeiro and Sebastiao, 2010). Although both A_1 and A_{2A} receptors are known to function in isolation as monomers, they are predominantly present as either homodimers or heterodimers, where they can form functional subunits with dopamine receptors (Ferre et al. 2016a, Ferre et al. 2016b; Ballesteros-Yáñez et al. 2018). The binding of caffeine to adenosine receptors results in dopamine release within the reward centres of the mammalian brain (Borycz et al. 2007; Ferré, 2016).

Caffeine not only functions pre- and post-synaptically at the synapse level but also permeates neurons to act intracellularly (Mustard et al. 2014). Intracellular targets of caffeine include ryanodine receptors (RyR), phosphodiesterases (PDE), and acetylcholinesterases (Mustard et al. 2014). Additionally, caffeine is known to modulate $GABA_A$ receptors and glycine receptors (Mustard et al. 2014). Although caffeine is known to act on a range of molecular targets, caffeine's effects in mammals are believed to be primarily mediated by adenosine receptors as concentrations required to antagonise these alternative targets in mammals would require toxic levels of caffeine consumption (Fredholm et al. 1999).

To date, only three adenosine receptors have been characterised in invertebrates. *Drosophila melanogaster* has a single adenosine receptor, *DmAdoR*. This receptor shares only 38 % identity to the human A_{2A} receptor (Kucerova et al. 2012). The starfish (*Asterina miniata*) has a single adenosine receptor with 48 % identity to the human A_1 receptor (Kalinowski et al. 2003). Additionally, the diamondback moth (*Plutella xylostella*) expresses a single adenosine receptor, PxAdoR, with 35 % identity to the human A_{2A}

receptor (Fang et al. 2016). All insect adenosine receptors exhibit close identify to the remaining human adenosine receptors (*D. melanogaster*: A_1: 36 %, A_{2B}: 35 % and A_3: 35 %; *P. xylostella*: A_1: 33 %, A_{2B}: 32 % and A_3: 34 %). Given the close homology of insect adenosine receptors to all known human adenosine receptor subtypes, makes it difficult to infer functionality and potential ligand specificity from homology alone (Pearson, 2013). Indeed, studies assessing the ligand specificity of *D. melanogaster* have identified that *DmAdoR* responds to adenosine but not to caffeine and other simple xanthine ligands (Kucerova et al. 2012), indicating the clear necessity of ligand characterisation of invertebrate adenosine receptors if these animals are to be used to study the addictive-like properties of this drug. Importantly, pharmacological studies have indicated that, in contrast to *Drosophila*, both the honeybee and the nematode have adenosine receptors that interact with caffeine directly (Wright et al. 2013; Bridi et al. 2015) in line with mammalian models, indicating that certain invertebrates may be more suited for the study of caffeine addiction than others. In addition, recently, evidence for an A_{2A}-D_2 receptor has been observed in the nematode (Manalo and Medina, 2018), indicating that oligomerisation of adenosine receptors is likely conserved across phyla, further supporting the use of invertebrates in the study of caffeine addiction.

Similar to mammals, insects are known to have RyR, PDEs, acetylcholinesterases, $GABA_A$ receptors, and glycine receptors (Mustard et al. 2014; Frenkel et al. 2017). Interestingly, it has recently been identified in the cricket that caffeine may function at these molecular substrates at significantly lower concentrations than observed in mammals (Sugimachi et al. 2016), indicating that alternative caffeine targets in certain insects may be substantially more sensitive to lower concentrations of the drug, however, it remains to be seen whether this sensitivity extends to all insects, or if this sensitivity is unique to crickets.

1.8.2 Modelling the stages of caffeine addiction in animal models: Lessons from mammals and insects

In contrast to research into other addictive compounds, research into caffeine has received considerably less attention in mammalian models of addiction, primarily due to the fact that repeated consumption of caffeine in most instances is not considered harmful

to either the individual or society (Satel et al. 2006). Studies have identified that primates will voluntarily self-administer intravenous caffeine injections; however, in contrast to other addictive compounds, injections tend to be sporadic and irregular rather than repeatedly and regularly self-administered (Deneau et al. 1969; Hoffmeister and Wuttke, 1973; Griffiths et al. 1979; Sekita et al. 1992). However, pre-exposure to caffeine (i.e. forced repeated injections) prior to the self-administration paradigm can generate self-administration in some instances (Deneau et al. 1969). Similar variable rates of intravenous self-administration have also been observed in the rodent literature (Collins et al. 1983; Briscoe et al. 1998), however again, pre-exposure protocols have been shown to lead to caffeine self-administration in some instances (Atkinson and Enslen, 1976). Collectively this suggests that, in contrast to nicotine, caffeine has only weak reinforcing effects in mammals.

Given that the delivery method of addictive compounds can profoundly alter both the pharmacokinetics and addictive potential of the compound (Allain et al. 2015), other studies have instead focused on oral self-administration of caffeine, given that this is the typical route of delivery in the general population (Fredholm et al. 1999). However, these studies have been shown to be confounded by the bitter taste of the compound, and many animals reject caffeine solutions in a dose-dependent manner (Vitiello and Woods, 1975; Tordoff et al. 2008; Field et al. 2010; Aspen et al. 1999; Vautrin et al. 2005; Kennedy et al. 2015). Thirst will force animals, however, to consume caffeine in water if no other water source is available (Vitiello and Woods, 1975; Field et al. 2010; Aspen et al. 1999; Vautrin et al. 2005).

Remarkably, only a handful of studies exist which have assessed whether oral preferences for caffeine can arise in rodent models. Intermediate-long continual oral access models (6-48 h) have shown that although hamsters display a preference for oral caffeine over vehicle alone (Frank et al. 2004), rats do not display a preference for a range of caffeine concentrations over a 48 h two-way choice period (Tordoff et al. 2008). However, continuous pre-exposure protocols (caffeine-laced water provided as the animals only water access for 10-14 days) have identified that rats display a preference for caffeine over vehicle alone in a dose-dependent manner in subsequent choice tests (Vitiello and Woods, 1975; Newland and Brown, 1992). Similarly, intermittent pre-exposure schedules of

caffeine administration (forced caffeine administration on alternate days for a period of 6 days) results in a preference for a caffeinated solution in subsequent two-choice tests; however, this preference is only observed if the bitter taste of caffeine has been 'masked' with sodium benzoate (Vautrin et al. 2005; El Yacoubi et al. 2005). Other studies have instead relied on masking the bitter taste of caffeine using a glucose/saccharin vehicle which can generate a preference for caffeine in some instances (Falk et al. 1999).

Studies assessing caffeine tolerance in mammals have identified that tolerance, here defined as an attenuation of the heightened locomotor response, in line with the psychomotor stimulant theory of addiction, is apparent within 3-5 days in rodents following chronic oral administration (Holtzman et al. 1983; Chou et al. 1985; Holtzman et al. 1988). Dependence has also been observed in rodents following chronic oral administration of caffeine for 14 days and leads to a blunting of the dopaminergic response to the drug following acute challenge (Quarta et al. 2004). In addition, chronic oral caffeine exposure has been shown to lead to upregulation of A_1 receptors in multiple brain regions in as little as 3 days (Ramkumar et al. 1988; Daval et al. 1989; Shi and Daly, 1998; Fredholm, 1982; Johansson et al. 1997). Negative reinforcement is common, and withdrawal symptoms are known to promote caffeine self-administration in a bid to alleviate the symptoms of withdrawal (Griffiths and Woodson, 1988; Hughes et al. 1991; Hughes et al. 1992; Hughes et al. 1995; Addicott and Laurienti, 2009).

To date, only three studies have identified a preference for caffeine in invertebrate models using short-access paradigms. Studies in honeybees have shown that free-flying honeybees presented with a choice between ~130 μM caffeine in sucrose or sucrose alone, consumed a greater total volume of the caffeinated solution over a 1 h period (Singaravelan et al. 2005). In contrast, Liao et al. (2017) identified that free-flying honeybees offered a choice for very low caffeine concentrations (0.5-5 μM), instead find caffeine aversive over a 2 h period. Studies in the nematode, *C. elegans*, have shown that nematodes display a preference for 0.1 % caffeinated agar quadrants over caffeine-free agar during a 90 min choice period (Urushihata et al. 2016). Finally, although not directly assessing a preference for caffeinated solutions, a recent study in the honeybee has shown that caffeine functions as a cognitive enhancer during olfactory conditioning of the proboscis extension reflex

(Wright et al. 2013), indicating that caffeine is capable of modulating reward learning in this insect.

To date, no studies have assessed caffeine tolerance or dependence in an invertebrate model, nor have changes in adenosine receptor expression been assessed following caffeine treatment; thus, it is not clear whether caffeine is capable of altering these molecular substrates in an analogous fashion in invertebrate models. Interestingly, a single study has identified that acute thoracic application of caffeine leads to an upregulation of dopamine receptors in the brain of the honeybee (Kucharski and Maleszka, 2005), indicating that caffeine interacts with dopaminergic signalling in this insect. Similarly, acute caffeine administration in mice has been shown to lead to dopamine receptor upregulation (Stonehouse et al. 2003), indicating that there is some conservation of changes in receptor expression across phyla.

1.9 Conclusion and project outline

Mammalian models of substance abuse have proved invaluable in identifying the neural substrates and pathophysiology involved in drug addiction, yet, our understanding of how these drugs lead to addiction remains incomplete (Huang et al. 2018). Despite having a substantially simpler nervous system to that of the mammal, insects utilise dopamine as the primary neurotransmitter for encoding reward and show maladaptive behaviours following consumption of a range of alkaloids known to function as addictive agents in mammals. Insects may therefore provide a "stripped-down" model for the study of addiction, removing layers of complexity and revealing the critical factors involved in the generation of addiction. Indeed, despite the insects substantially simplified nervous system, previous studies have shown that insects can serve as effective models for a range of complex neuropathologies, such as Alzheimer's disease (Prüßing et al. 2013), Parkinson's disease (Xiong and Yu, 2018), autism (Tian et al. 2017), schizophrenia (Furukubo-Tokunaga, 2009), attention deficit hyperactivity disorder (Lebestky et al. 2010), and depression (Ries et al. 2017), indicating the utility of these animals in the study of complex disorders.

Although *Drosophila* has historically been considered the insect "workhorse" for the modelling of complex brain diseases, bees may be particularly advantageous to the

study of addiction due to their rich behavioural repertoire (Chittka and Nivan, 2009), remarkable ability for associative memory (Menzel and Muller, 1996), and ready amenability to electrophysiological recordings (Strube-Bloss and Rössler, 2018). Furthermore, although genetic manipulation techniques in *Drosophila* surpass the techniques currently available in the bee, recent advances have been made in both genetic and transcriptomic manipulation techniques in honeybees (Antonio et al. 2008; Wang et al. 2013; Kohno et al. 2016; Wenfeng et al. 2016; Costa et al. 2016). The ability to manipulate key receptors and molecular pathways involved in reward learning in the bee further indicates the potential utility of these insects in identifying the underlying mechanisms responsible for the generation of addiction.

Although numerous studies have identified addictive-like behaviour to a range of substances in invertebrate species (Wolf and Heberlein, 2003; Kaun et al. 2012; Søvik and Barron, 2013; Landayan and Wolf, 2015; Ryvkin et al. 2018; Lowenstein and Velazquez-Ulloa, 2018), at present, studies assessing the viability of insects to model nicotine and caffeine addiction are very limited. Of those that exist in the bee, few have systematically compared the propensity to display addictive-like behaviour across bee species. In addition, no studies to date have assessed whether pre-exposure or intermittent schedules of drug administration will affect the insect's motivation to consume these alkaloids. Given that the schedules of drug administration are critical in identifying whether mammals display addiction-like behaviour to nicotine or caffeine, this represents a clear gap in our current understanding.

This thesis will examine whether bumblebees and honeybees may function as suitable models for the study of nicotine and caffeine addiction. It will look to identify whether there is an optimum species, compound concentration, and schedule of administration that results in preferential consumption of these alkaloids in a caged setting within a laboratory. Specifically, this thesis will primarily address whether bees alter their consumption of nicotine or caffeine following forced chronic or intermittent pre-exposure to either compound when delivered in their food. I predict that bees will be more likely to preferentially consume either alkaloid when they have consumed the compound over a number of days, in comparison to unexposed bees, in line with what is observed in mammalian models of addiction. Furthermore, given that these alkaloids are known to be

perceived as bitter, and as the background sugar that alkaloids are delivered in is known to offset this bitterness, I will also assess whether altering the background sugar concentration affects the bee's propensity to consume these compounds. I predict that bees will be more likely to avoid or be indifferent to alkaloids when presented in a low sucrose background in comparison to when bees are presented identical alkaloid concentrations in a high sucrose background. Finally, given that addiction is defined by evidence of aberrant behaviour, I will assess whether bees that have been pre-exposed to nicotine will make sub-optimal choice behaviour in order to continue their consumption of nicotine, in line with the DSM-V and ICD10 criteria for addiction. I predict that if nicotine exerts addictive properties on bees, then bees would display a preference for nicotine presented in a sucrose solution over sucrose of a higher molarity.

Chapter 2.0 General Methods

2.1 Capture and Restraint

All experiments were performed at Newcastle University, Newcastle upon Tyne. Forager honeybees (*Apis mellifera, buckfast or carnica*), approximately 3 weeks old, were collected between May - October each year from 2015-2017 from six free-flying outdoor colonies. The colonies were originally obtained from the UK's National Bee Unit (Sand Hutton, Yorkshire). A wire pollen trap mesh was placed at the hive entrance to slow the bees re-entry. Honeybees were collected using small glass phials (5 bees per phial). Only bees returning to the hives were collected to ensure foraging status.

Bumblebees (*Bombus terrestris audax*) colonies (Koppert UK Ltd.) were maintained in laboratory conditions (22-27°C and 60-70 % RH) in continuous darkness, and were provided with *ad libitum* commercial sugar water and pollen mix (Pollen mix, Koppert UK Ltd.) prior to experimentation. Individual bees were collected in a small glass phial as they exited the colony. Worker bees were sexed (by identification of a stinger), and their thorax width measured prior to experimentation. Only female bees with a thorax width >4.5 mm were used in experimentation to minimise the likelihood of inclusion of nurse bees (Goulson et al. 2002).

Honeybees and bumblebees were cold anaesthetized on ice for approximately 3 min, or until movement slowed sufficiently, before being placed into feeding cages. Cages were plastic boxes (145 x 130 x 60 mm) with 1 mm ventilation holes. Additional 10 mm holes were positioned on either side of the cage where feeding tubes could be inserted horizontally (Fig. 2.1). Feeding tubes were modified 2.5 ml Eppendorf tubes with four 4 mm equidistant holes allowing access to the solution within. Bees were allowed to feed from these tubes *ad libitum* during experimentation. A piece of paper was placed in the bottom of the cage and replaced when needed to allow removal of bee defecation, maintaining hygienic conditions. Following a 1 h period of acclimation to the caged environment, feeding tubes were inserted into the feeding cages, and the cages were placed in an incubator (Sanyo/Panasonic) for the duration of the feeding assays. The incubators were maintained at 60 % RH, under constant darkness, at a constant temperature of 34°C and 28°C for honeybees and bumblebees, respectively, mimicking hive conditions

(Heinrich, 2004). Honeybees were housed in cohorts of 20 bees, whereas bumblebees were housed individually. All bees were randomly assigned to a treatment.

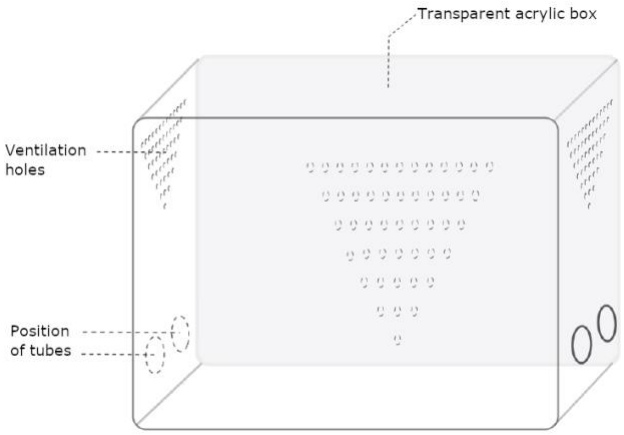

Figure 2.1 | Cages used in the behavioural experiments. Plastic cages in which 20 honeybees or individual bumblebees were placed for the pre-exposure periods and 24 h two-way choice assays.

2.2 Solutions

Sucrose solutions were made in deionised water, and stock solutions of nicotine and caffeine were prepared by dissolving either alkaloid within a sucrose solution. Sucrose concentrations were 0.5 M, 1.0 M, 1.1 M, 1.2 M, 1.3 M, or 1.5 M, depending on the experiment. A 25 mM stock solution of ((-)-Nicotine hydrogen tartrate salt (Sigma Aldrich) and a 25 mM stock solution of caffeine (Sigma Aldrich) were made and stored at -20°C until required. All working solutions were prepared by serial dilution and stored in the fridge at 4°C. Fresh working solutions were made on a weekly basis. All sucrose and alkaloid concentrations used, and the justification for their use is detailed within individual chapters.

2.3 Feeding assays

2.3.1 Unexposed bees: 24 h two-way choice assay

In the unexposed 24 h two-way choice experiments, bees were given a choice between two solutions immediately following capture and restraint (Fig. 2.3.1). Cohorts of 20 honeybees or individual bumblebees were offered a choice between either sucrose alone, or equimolar sucrose containing a specific concentration of either nicotine or

caffeine. To measure the consumption of each solution, individual tubes were weighed prior to insertion, and again 24 h later. Cages with feeding tubes but without bees were used to control for evaporation. Consumption was measured as the difference in weight after the 24 h period, minus evaporation. This was divided by the density of the solution (1.062 for 0.5 M sucrose, 1.127 for 1 M sucrose, and 1.192 for 1.5 M sucrose. Densities for 1.1 M, 1.2 M, and 1.3 M are listed below) to convert mass values to volume. For honeybees, this was further divided by the number of honeybees alive in the cage at the end of the 24 h period to obtain the final volume consumed per bee. All tube positions were counterbalanced to remove any positional bias.

2.3.2 Three and five day pre-exposure assays

In the 3 day and 5 day pre-exposure period bees were provided with either alkaloid continuously (i.e. no choice was provided) for a period of 72 h or 120 h. Following this, bees were presented with a 24 h two-way choice between either sucrose alone or equimolar sucrose plus the previously administered concentration of the alkaloid; e.g. bees that had been provided with 25 μM nicotine in 0.5 M sucrose for 3 days were presented with a choice between 25 μM nicotine in 0.5 M sucrose, or 0.5 M sucrose alone (Fig. 2.3.1). Dead honeybees and bumblebees were counted daily to monitor mortality throughout the assay. To record the rate of consumption across time during the pre-exposure period, feeding tubes were weighed and replaced after every 24 h period and total consumption calculated as described previously.

2.3.3 Intermittent treatments: Honeybees only

Honeybees were presented with three different intermittent feeding schedules: 12 h intermittent (I12), 48 h intermittent (I48), or extended 48 h intermittent (EXT48). I12 bees were provided with sucrose containing either alkaloid daily between 9 am and 9 pm, and sucrose alone between 9 pm and 9 am for a period of 72 h (Fig. 2.3.1). I48 bees were provided with sucrose containing either alkaloid for a period of 48 h, followed by sucrose alone for 24 h (Fig. 2.3.1). The EXT48 schedule of administration was only conducted for caffeine. Here, bees were provided with caffeine for a period of 48 h, followed by sucrose for 24 h, then received a further 24 h administration of caffeine and a final 24 h of sucrose alone (Fig. 2.3.1). After the intermittent feeding schedules, bees were presented with a 24

h two-way choice between either sucrose alone, or equimolar sucrose plus the previously administered concentration of the alkaloid. Dead bees were counted daily. To record the rate of consumption across time during the pre-exposure period, feeding tubes were weighed and replaced after every 12 h (I12) or 24 h (I48 and EXT48) period, and total consumption was calculated as described previously.

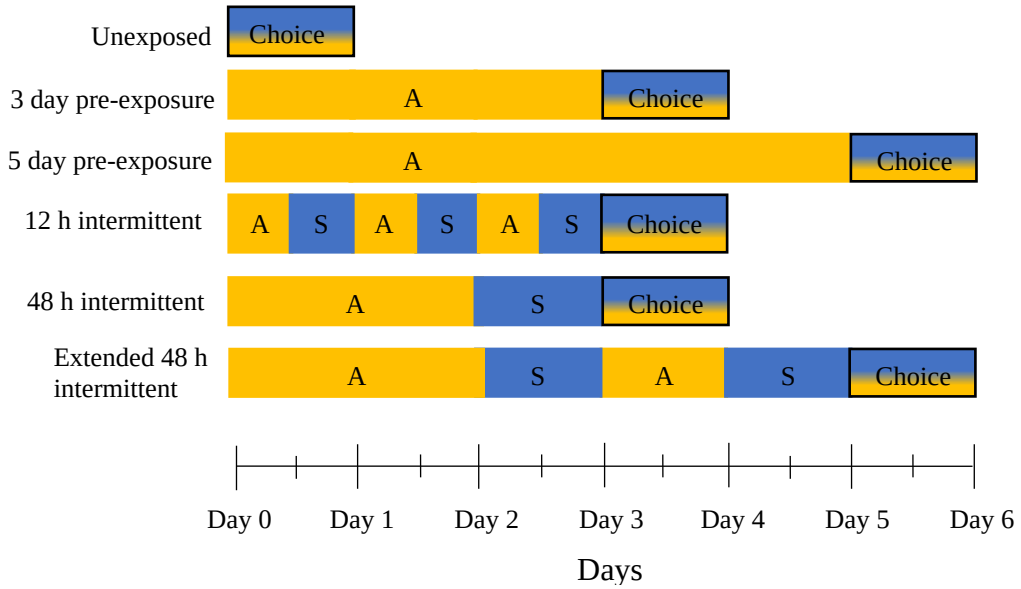

Figure 2.3.3| Feeding schedules used in honeybee and bumblebee choice experiments. A (Alkaloid: Yellow) represents periods where bees had access to either alkaloid provided in a sucrose solution. S (Sucrose: Blue) represents periods where bees had access to sucrose alone. Choice (24 h two-way choice) represents the choice period where bees were presented with either sucrose alone, or sucrose containing the previously administered concentration of the alkaloid.

2.3.4 Valence experiment: Bumblebees only

In the valence experiment, bumblebees were pre-exposed to either sucrose alone (control) or 100 µM nicotine dissolved in 1.0 M sucrose continuously (treatment) for a period of 72 h. Following this, treatment bees were presented with a 24 h two-way choice between either 1.0 M, 1.1 M, 1.2 M, or 1.3 M sucrose, or 1.0 M sucrose plus 100 µM nicotine. Bumblebees in the control group were offered 1.0 M sucrose alone, or a choice between 1.0 M, 1.1 M, 1.2 M, or 1.3 M sucrose (Fig. 2.3.2). Consumption throughout the chronic feeding and choice period were calculated as described previously (Density of sucrose solutions: 1.142 for 1.1 M, 1.156 for 1.2 M, and 1.166 for 1.3 M).

At the end of all experiments, bees were euthanized in a -80^{o}C freezer.

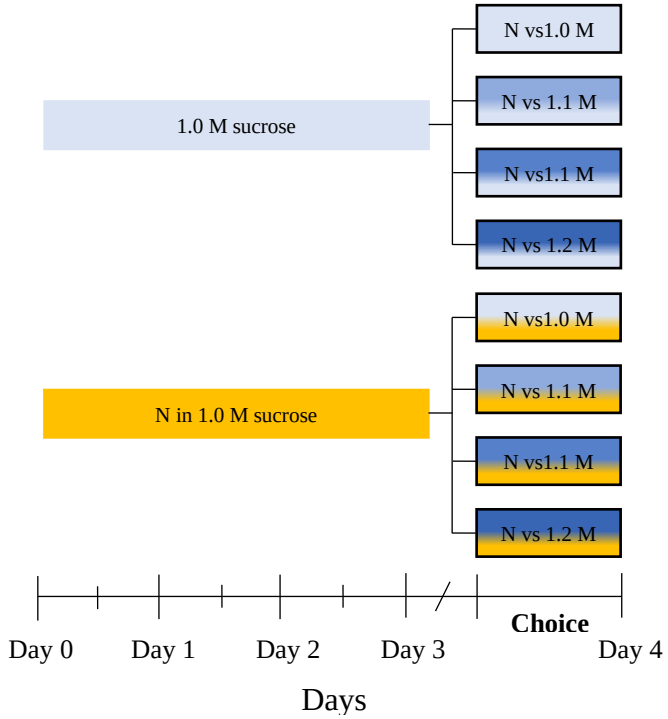

Figure 2.3.4| Feeding schedules used in honeybee and bumblebee choice experiments. Eight treatments were conducted in total. Nicotine (N: Yellow) represents periods where bees had access to 100 µM nicotine dissolved in a 1.0 M sucrose solution. Sucrose (Blue) represents periods where bees had access to sucrose alone. Choice (24 h two-way choice: yellow/blue and blue boxes) represents the choice period where bees were presented with either sucrose alone (control choice: 1.0 M sucrose vs 1.0 M sucrose, 1.0 M sucrose vs 1.1 M sucrose, 1.0 M sucrose vs 1.2 M sucrose, 1.0 M sucrose vs 1.3 M sucrose), or 1.0 M sucrose containing the previously administered concentration of nicotine versus a higher molarity sucrose solution (Treatment choice: 100 µM nicotine in 1.0 M sucrose vs 1.0 M sucrose, 100 µM nicotine in 1.0 M sucrose vs 1.1 M sucrose, 100 µM nicotine in 1.0 M sucrose vs 1.2 M sucrose, 100 µM nicotine in 1.0 M sucrose vs 1.3 M sucrose).

2.4 Statistical methods

All data were analysed using IBM SPSS (Version 23). Statistical methods used and the critical assumptions for these tests are summarised in table 2.4. Normality was assessed using the Shapiro-Wilks test for normality. If data did not display a normal distribution, the data were square-root or log10 transformed. If the assumption of normality was still not met following transformation, non-parametric models were applied to the original un-transformed data set. Any instances of data transformation are detailed within the extended data tables. All values are reported to 3 significant figures with the exception of partial eta squared and *p* values which are presented to 3 decimal places unless the value is < 0.001; the level of precision allowed by SPSS.

To analyse whether the bees total food consumption varied as a function of time or treatment across the pre-exposure periods, repeated-measures general linear models were conducted (RM-GLM). Instances where the assumption of sphericity has not been met and corrections were used are reported within the extended data tables.

To analyse whether bees displayed a preference for either alkaloid in the 24 h two-way choice tests, data was indexed with positive values indicating a preference for the alkaloid containing solution and negative values a preference for the sucrose solution (i.e. an aversion to the alkaloid) (Index calculation: solution 1 volume – solution 2 volume) / (solution 1 volume + solution 2 volume). A value of 0 represents no preference for either solution (i.e. bees ate equally from both tubes). For control treatments, tubes were labelled 'A' and 'B' and indexed as detailed above. As no preference was expected for either tube in the control treatment, control values are expected to be approximately 0. General linear models (GLM) or generalised linear models (GzLM) with a linear distribution were conducted to assess whether preference varies as a function of pre-exposure or nicotine concentration. Additionally, *post-hoc* one-sample t-tests against 0 were conducted to identify if the indexed values were significantly different to zero. Where appropriate, GLM and GzLMs have been followed with *post hoc* analyses using least significant differences (LSDs), Bonferroni, or Holm-Bonferroni corrections.

To analyse whether total consumption during the 24 h two-way choice day varied as a function of pre-exposure or concentration, GLMs or GzLMs were conducted. In order to assess whether total consumption varied within treatments from their respective controls, one-way analysis of variance (ANOVA) was conducted. If data did not meet normality and could be not be transformed, Kruskal Wallis tests were performed. For treatments where there are only two concentrations being compared, independent samples T-tests were performed. Where the assumption of normality was not met and the data could not be successfully transformed, Mann-Whitney U tests were performed instead.

The dose that the bees consumed is defined as the amount of an alkaloid consumed over a set period of time and represents the absolute dose. Dose consumed during the 24 h two-way choice day was calculated by the average consumed within the 24 h period from

the alkaloid tube alone (presented as μg/bee/day or ng/bee/day). This value was calculated as the product of the μg/ml or ng/ml of the alkaloid in the food solution and the amount of solution consumed (μl) per bee during the 24 h period. Average dose over the 3 day or 5 day pre-exposure periods were calculated as the combined average dose consumed over the 24, 48, 72, 96, and 120 h consumption periods. The combined average dose across the pre-exposure period was chosen as total consumption was shown to vary across the pre-exposure period, making selection of a single pre-exposure dose based on a single time point inappropriate. For the bumblebee dose data, 95 % confidence intervals on the dose consumed are provided. The average dose consumed was analysed both within and between treatments. To analyse whether total dose increased as a function of alkaloid concentration within treatments, Welch's ANOVAs were conducted with *post-hoc* Games-Howell tests. These tests were chosen as all data sets did not meet the assumption of homogeneity of variance (HOV). To analyse whether the chosen dose was statistically different depending on whether bees had been pre-exposed to the alkaloid prior to the choice day, generalised linear models (GzLM) were conducted. The GzLM was set to a gamma distribution; this distribution was chosen as these data sets were positively skewed. GzLMs with gamma distributions were also conducted to analyse whether the dose consumed during the pre-exposure period was statistically significant to the dose chosen during the 24 h two-way choice.

Mortality in honeybees was assessed using Kruskal-Wallis tests, and where appropriate, followed with *post hoc* Mann-Whitney U tests. Mortality in bumblebees was assessed using binary logistic regression. In all cases, mortality was assessed as the difference between the number of bees alive at the start of the experiment in comparison to the number of bees alive at the end of the experiment.

Note that due to human error over the course of the pre-exposure period, (i.e. spilt feeding tubes at a particular time point), any affected data sets were removed from the analysis of total consumed over the pre-exposure period, to allow for a repeated measures design. All other data (i.e. indexed preference, total consumption over the 24 h choice period, and dose consumed during the choice days) represents the complete data. As such, on occasion, there is a slight variation in sample size between the pre-exposure period and

the remaining data. These variations are infrequent, and all sample sizes are detailed in the extended data tables.

Table 2.4 | Summary of statistics used and the critical assumptions. ANOVA (Analysis of variance), GLM (general linear model), GzLM (Generalised linear model), HOV (Homogeneity of variance), RM-GLM (Repeated measures GLM).

Experimental data	Test	Assumptions	Test
Total consumption across the pre-exposure period	RM-GLM	Normality HOV Sphericity	Shapiro-Wilk Levene's test Mauchly's test
24 h two-way choice tests for preference	(i) GLM	Normality HOV	Shapiro-Wilk Levene's test
	(ii) GzLM	N/A	N/A
	(iii) One sample T-tests against zero	Normality	Shapiro-Wilk
Total consumption during the 24 h two-way choice period	(i) GLM	Normality HOV	Shapiro-Wilk Levene's test
	(ii) One-Way ANOVA	Normality HOV	Shapiro-Wilk Levene's test
	(iii) Independent samples T-test	Normality HOV	Shapiro-Wilk Levene's test
	(iv) Kruskal Wallis tests	N/A	N/A
	(v) Mann-Whitney U test	N/A	N/A
Comparing dose consumed between concentrations within the level of treatment	Welch's ANOVA with *post-hoc* Games-Howell	Normality	Shapiro-Wilk
Comparing dose consumed between treatments or time	GzLM (Gamma distribution)	N/A	N/A
Mortality (Honeybees)	Kruskal Wallis	N/A	N/A
Mortality (Bumblebees)	Binary logistic regression	N/A	N/A

Chapter 3.0 Do honeybees prefer to consume nicotine delivered in a sucrose solution over sucrose alone?

3.1 Introduction

Nicotine is a potent alkaloid found in the flowers, nectar, and pollen of *Nicotiana* spp. (Singaravelan et al. 2005; Adler et al. 2006; Tadmor-Melamed et al. 2004; Kessler et al. 2010). It is also the primary psychoactive agent within cigarette smoke (Benowitz, 2010). In low doses, nicotine improves cognitive performance in humans (Wignall and de Wit, 2011), improves memory consolidation (Beer, 2016), alleviates depression (Powledge, 2004), functions as an anxiolytic (Anderson and Brunzell, 2015), and induces mild euphoria (Agué, 1973; Benowitz, 1988). Despite the acute beneficial effects of nicotine, repeated administration of the drug typically results in the development of addiction (Benowitz, 2010). Indeed, globally, more people are addicted to nicotine than to any other drug (Peacock et al. 2018). Despite this, nicotine is the least studied addictive agent (Ahmed, 2010), and the molecular and neurological alterations that result in the development of nicotine addiction are far from clear (Benowitz, 2010; Brunzell et al. 2015).

Nicotine is believed to have evolved primarily as a natural pesticide to deter herbivores (Steppuhn et al. 2004). Nicotine is both bitter and toxic to animals in high doses (Collins et al. 2012; Lachenmeiera and Rehm, 2015), and the concentrations of nicotine that are present in the leaves, seeds, and flowers of the plant are known to deter herbivorous insects (Steppuhn et al. 2004; Kessler et al. 2008). Given the clear role of nicotine as a defence mechanism against insects, it was unexpected to find that low, non-toxic concentrations of nicotine are present in floral rewards given to pollinators (nectar and pollen) (Detzel and Wink, 1993; Singaravelan et al. 2005; Adler et al. 2006; Tadmor-Melamed et al., 2004; Kessler et al., 2010). These studies indicate a potential role for nicotine in mediating plant-pollinator interactions. Indeed, free-flying honeybees have been shown to exhibit a preference for sucrose solutions containing low concentrations of nicotine (Singaravelan et al. 2005), and injections of nicotine into the brain of honeybees have been shown to improve memory retention (Thany and Gauthier, 2005). In addition, studies in *Drosophila* have indicated that nicotine is capable of stimulating dopamine efflux through direct interaction with nicotinic acetylcholine receptors in the insect brain

(Pyakurel et al. 2018; Shin and Venton 2018). This suggests that the impact of nicotine on the insect brain is analogous to its action on the mammalian brain (McCallum et al. 2006; Tammimäki et al. 2006; Leino et al. 2018), perhaps indicating that insects could also become addicted to the compound. In an ecological context, this could strengthen the association between floral cues and the bees memory of floral rewards, resulting in either enhanced pollination for *Nicotiana* spp. that are pollinated by bees (Ollerton et al. 2012), or potentially reducing the plants' reproductive fitness in the case of bees that nectar-rob from these plants (Ollerton et al. 2012).

Mammalian studies of nicotine addiction have established that rodents exhibit only a passive interest to self-administer nicotine when first presented with the opportunity to self-administer the drug (Valentine et al. 1997; Fu et al. 2001; Brower et al. 2002; Fu et al. 2003; Parker et al. 2004; Peartree et al. 2012; Yan et al. 2012). However, rodents will gradually increase their self-administration of nicotine over time if allowed prolonged periods of access to the drug (~7-14 days) (Valentine et al. 1997; Fu et al. 2001; Brower et al. 2002; Fu et al. 2003; Parker et al. 2004; Peartree et al. 2012; Yan et al. 2012). In addition, the development of preferential nicotine administration can be shortened or enhanced by introducing a 'priming' procedure, whereby rodents undergo a period of forced nicotine administration prior to self-administrative sessions (Shoaib et al. 1997; Adriani et al. 2003; Natividad et al. 2013; Neugebauer et al. 2014; Renda and Nashmi, 2014). Finally, intermittent access paradigms, whereby rodents undergo periods of imposed abstinence to the drug, have been shown to lead to enhanced nicotine self-administration upon drug reintroduction (George et al. 2007; Cohen et al. 2012; Cohen et al. 2013; Gilpin et al. 2014; Cohen et al. 2015). Collectively, these studies indicate that the duration and frequency of nicotine access are critical in the development of nicotine addiction in rodents. The fact that rodents exhibit only a passive interest to self-administer nicotine when first presented with the drug, indicates that nicotine has only weak positive reinforcing properties in these animals. However, the fact that animals will gradually increase their self-administration of nicotine over time, indicates that nicotine's positive reinforcing effects are sufficient to result in self-administration in rodents. The fact that preferential nicotine administration can be shortened or enhanced by introducing a priming procedure, indicates that rodents can be forced into an addicted state. This occurs as priming procedures facilitate neurological changes in advance of self-administration protocols, resulting in a rapid state

of nicotine dependence (Damaj et al. 2003; Grabus et al. 2005 Gould et al. 2012). Thus, if animals increase their self-administration of a compound following a priming procedure (i.e. following pre-exposure), then this suggests that the animal can be forced into an addicted state, and suggests that neurological changes are occurring during this pre-exposure period to account for this behaviour. Finally, the enhanced nicotine self-administration that is observed in intermittent protocols is thought to reflect periods of pronounced negative reinforcement caused by the avoidance of withdrawal (George and Koob, 2017). Withdrawal serves as evidence of the animal being in an addicted state, in line with the DSM-V and ICD10 criteria for diagnosing addiction (see 1.5). Therefore, if an animal exhibits enhanced nicotine self-administration following an intermittent nicotine administration protocol, then this is suggestive of the animal experiencing withdrawal from the compound. Although it is not essential that an insect model comprehensively shares all of the responses observed in rodent model paradigms, evidence of priming procedures facilitating nicotine self-administration, and imposed abstinence facilitating increased nicotine self-administration, would serve to support the validity of using insects to model addiction.

At present, only a single study has assessed whether honeybees perceive nicotine as rewarding. Singaravelan et al. (2005) identified that free-flying honeybees preferentially consume low concentrations of nicotine in a sucrose solution over sucrose without nicotine, which suggests that nicotine functions as a positive reinforcer in these insects. However, these studies only looked at nicotine access over a short period of time (1 h). To date, no studies have examined whether honeybees display a preference for the compound when nicotine access is present over an extended period (24 h), or following nicotine pre-exposure, either chronically or in an intermittent fashion. Here, it was tested whether forced chronic pre-exposure or intermittent schedules of pre-exposure affect an observed preference for nicotine-containing food in groups of adult worker honeybees. In addition, previous studies have identified that honeybees find high concentrations of nicotine aversive (Köhler et al. 2012a); however, their aversion to nicotine can be offset by increasing the concentration of sucrose that the compound is administered in. Therefore, I also assess whether the concentration of sucrose that nicotine is administered in can affect the honeybees consumption of nicotine-containing food.

3.2 Methods

Honeybees were exposed to five different feeding schedules in these experiments: (i) Unexposed, (ii) pre-exposed for 3 days, (iii) pre-exposed for 5 days, (iv) intermittent 12 h (I12), and (v) intermittent 48 h (I48) (see General methods). The concentrations of sucrose and nicotine used in these experiments are detailed in table 3.2. Nicotine concentrations for the 0.5 M sucrose unexposed and 3 day pre-exposure assay were selected as they encompass the concentrations of nicotine known to be both preferred and avoided in prior experiments in honeybees (see table 1.5.3). A range of nicotine concentrations are required in order to assess whether honeybees display a biphasic dose-response curve for nicotine in a caged setting within a laboratory. Biphasic response curves are typically observed in addiction studies and aid in identifying the ideal concentration with which to study addiction-like behaviour (see 1.7.2 & 6.3). Given that unexposed and pre-exposed honeybees were shown to avoid high concentrations of nicotine when delivered in 0.5 M sucrose, regardless of whether they had been pre-exposed to nicotine or not, all remaining studies used 25 μM or 100 μM nicotine as honeybees did not display a strong aversion to these concentrations, i.e. 250 μM and 500 μM nicotine were likely within the high end of a biphasic response curve, and were unlikely to be fruitful to future studies.

Sucrose concentrations for the unexposed and 3 day pre-exposure assays were selected as they encompass the total sugar concentrations found within *Nicotiana* spp. nectar (0.5 – 3.0 M) (Kessler et al. 2012; Tiedge and Lohaus, 2017; Tiedge and Lohaus, 2018), and concentrations within this range have been used previously in the literature (see table 1.5.3), allowing for comparisons with previous studies. In addition, previous studies have indicated that the honeybees aversion to food containing nicotine can be offset by increasing the concentration of sucrose that the compound is administered in (Köhler et al. 2012a). Modifying the sucrose concentration in this study, therefore, provided a means to modulate the bitter tastant effects of nicotine on the bees feeding behaviour. Although honeybees did not display an aversion for 25 μM or 100 μM nicotine, if bees perceived this concentration of nicotine as bitter, then this may influence their choice behaviour. Given the results obtained for the chronic pre-exposure assays in 0.5 M, 1.0 M, and 1.5 M sucrose, the intermittent assays used a sucrose concentration of 1.0 M, simply as this was the

midpoint between all sucrose concentrations used, and sucrose molarity did not appear to be a factor governing the honeybees choice behaviour in the 24 h two-choice tests.

Unexposed treatments were conducted once for each sucrose and nicotine concentration (table 3.2). In all cases, the unexposed experiments were run separately from the pre-exposed experiments; however, the experiments were conducted at the same time of year to account for seasonal variability, e.g. for the 1.0 M sucrose experiments, the unexposed treatments were conducted 2-4 weeks prior to the 3 and 5 day pre-exposure treatments. It was not possible to conduct the 1.0 M sucrose intermittent treatments at the same time as the 1.0 M sucrose unexposed and 1.0 M sucrose 3 day pre-exposed and 5 day pre-exposed treatments. As such, the intermittent treatments were conducted the following year, at the same time of year (high summer), and using the same hives that were used for previous 1.0 M sucrose treatments. The data obtained for the unexposed experiments is used to compare against all pre-exposure and intermittent exposure experiments, e.g. the data obtained for the 1.0 M unexposed treatment is used as a comparison to the 3 day pre-exposure, 5 day pre-exposure, and I12 and I48 treatments. To make it clear that the data obtained for the 1.0 M unexposed treatments is used as a comparison in the I12 and I48 treatment, this is represented graphically with dashed lines.

Table 3.24 | Nicotine concentrations (μM) and sucrose concentrations (M) used in the honeybee choice experiments. I12 (12 h intermittent), I48 (48 h intermittent).

Feeding Schedule	0.5 M sucrose	1.0 M sucrose	1.5 M sucrose
Unexposed	0, 25, 100, 250, 500	0, 25, 100	0, 25, 100
Pre-exposure: 3 day	0, 25, 100, 250, 500	0, 25, 100	0, 25, 100
Pre-exposure: 5 day	N/A	0, 25, 100	N/A
Intermittent: I12, I48	N/A	0, 25, 100	N/A

3.3 Results

3.3.1 The response of unexposed and pre-exposed honeybees to different concentrations of nicotine administered in 0.5 M sucrose

Honeybees consumed the solutions in a way that varied over the pre-exposure period as a function of both time and treatment (Fig. 3.3.1a. RM-GLM, Time x Concentration: $F_{(10.3, 306)} = 2.69$, $\eta_p^2 = 0.083$, $p = 0.003$. Extended data table 3.3.1.1a). In general, they consumed as much as 140 µl of solution on the first day; on the second day, they consumed approximately 55 µl less and maintained this lower rate of consumption for the final 2 days. The inclusion of 250 µM or 500 µM nicotine within the solution resulted in a reduction in total volume consumed in comparison to control bees (0 µM) over the first day of pre-exposure (Fig.3.3.1a. Extended data table 3.3.1.1a). On the second day of pre-exposure, all honeybees that had nicotine in their solution consumed a significantly lower total volume of solution than the control (Fig.3.3.1a. Extended data table 3.3.1.1a); however, all treatments consumed approximately equal volumes of solution during the final pre-exposure day and the choice day (Fig.3.3.1a. Extended data table 3.3.1.1a).

The data for the choice test show that exposure to nicotine in food for prolonged periods influenced choice behaviour (Fig 3.3.1b. GzLM Concentration as main effect: $\chi^2_{(4)} = 21.7$, $p = <0.001$. Extended data table 3.3.1.1b). Honeybees displayed no preference or aversion to solutions containing 25 µM or 100 µM nicotine (*post hoc* comparison against control (LSD), $p = 0.184$ and 0.963, respectively). In contrast, a significant aversion was observed for solutions containing concentrations of nicotine $\geq$ 250 µM; bees avoided these solutions regardless of whether they had been pre-exposed to nicotine (*post hoc* comparison against control (LSD), $p = 0.046$ and 0.009, respectively). Note, however, that the aversion to 250 µM nicotine in the 3 day pre-exposed bees did not reach significance when considered in individual *post hoc* comparisons (One-sample T-test against 0: Holm-Bonferroni adjusted, $t_{(24)} = -2.18$ $p = 0.280$). That is, bees displayed an aversion in comparison to control; however, bees did not overall consume a greater total volume from the sucrose-containing tube over the two-way choice period.

Honeybees: Three day pre-exposure to nicotine in 0.5 M sucrose

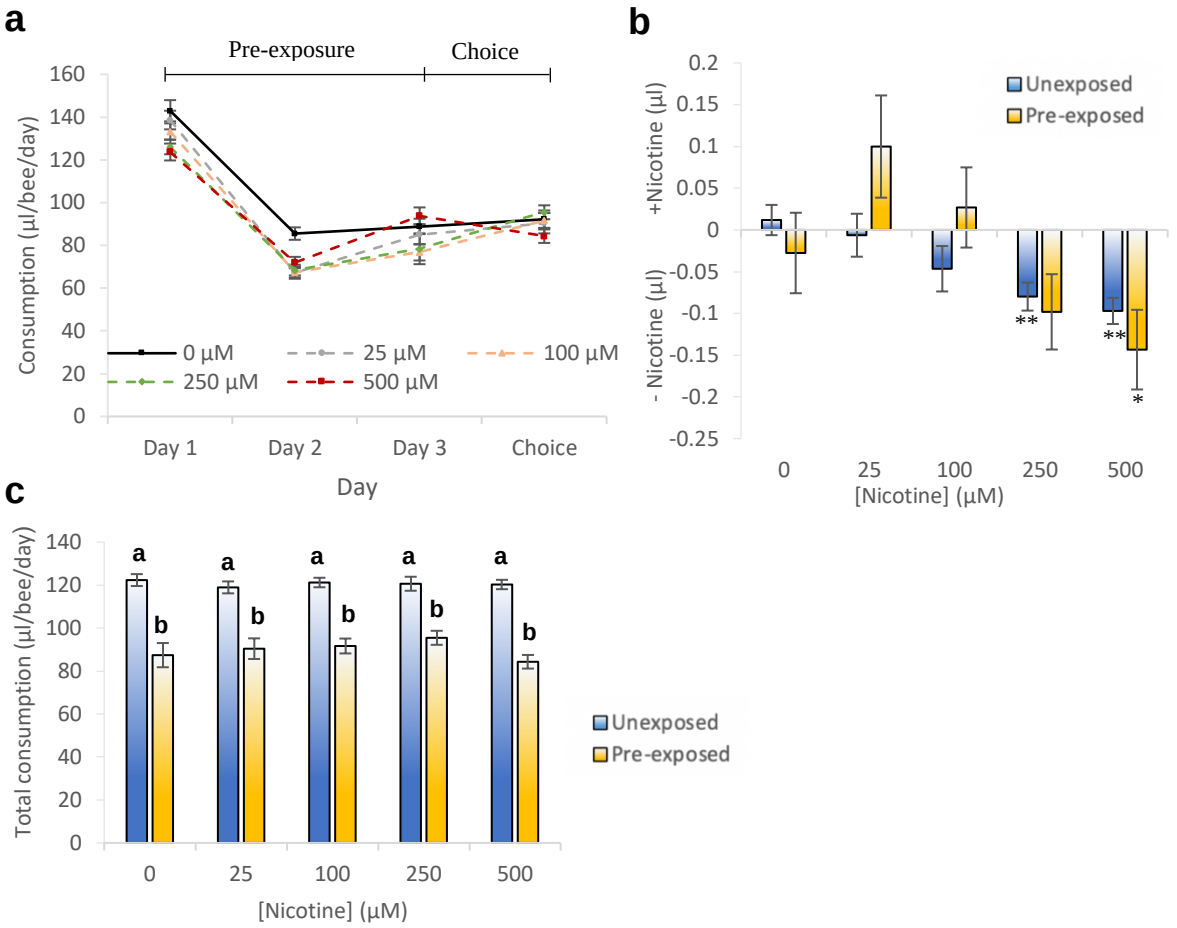

Figure 3.3.1 | Honeybees displayed an aversion for 250 µM and 500 µM nicotine in 0.5 M sucrose. **(a)** Pre-exposure to 25 µM, 100 µM, 250 µM, or 500 µM nicotine leads to a reduction in the total volume consumed across the 3 day pre-exposure period in comparison to sucrose alone (Extended data table 3.1.1.1a). **(b)** Honeybees displayed an aversion for 250 µM and 500 µM nicotine during the choice period (Extended data table 3.1.1.1b). Data represent the mean difference in the amount consumed over 24 h; positive values indicate a preference for nicotine and negative avoidance of nicotine. Asterisks indicate significant differences for one-sample t-tests against 0 (Holm-Bonferroni adjusted: *p <0.05; **p <0.01). Note that the aversion to 250 µM nicotine in the 3 day pre-exposed bees does not reach significance (250 µM $t_{(24)}$ = -2.18 p = 0.280). **(c)** Honeybees in the pre-exposed treatment group consumed a significantly lower total volume of solution during the choice period than the unexposed treatment group for all nicotine concentrations. However, on average nicotine-exposed bees did not consume less than their specific sucrose-only control group (Extended data table 3.3.1.1c). Letters indicate significant differences for *post hoc* comparisons between treatments at each concentration (Bonferroni adjusted). Bars and line graphs indicate mean (±s.e.m.) of consumption (µl) per bee, controlled for by evaporation.

Honeybees in the pre-exposed treatment group consumed a significantly lower total volume of solution during the choice period than unexposed bees for all nicotine concentrations (Fig. 3.3.1c. GzLM, main effect of Treatment: $\chi^2_{(1)} = 196$, $p = <0.001$). However, note that, on average, nicotine-exposed honeybees did not consume less than their specific sucrose-only control group during the choice period (Fig. 3.3.1a, 3.3.1c) (Extended data table 3.3.1.1c).

The dose of nicotine among the pre-exposed bees was as much 4-20x greater than the lowest concentration of nicotine, depending on the treatment (Dose table 3.3.1, Welch's ANOVA, $F_{(3,42.2)} = 1260$, $\eta_p^2 = 0.969$, $p = <0.001$. Extended data table 3.3.1.2). For example, over the pre-exposure period, honeybees that consumed 25 μM nicotine consumed a dose of 1.21 μg/bee/day, whereas those that consumed 500 μM nicotine consumed an average dose of 24.0 μg/bee/day, representing a 20-fold increase in the nicotine dose consumed by bees pre-exposed to 500 μM nicotine.

During the choice test, the pre-exposed honeybees chose a significantly lower dose of nicotine than the unexposed honeybees (GzLM, concentration x treatment, $\chi^2_{(3)} = 48.7$, $p = <0.001$. Extended data table 3.3.1.2d). In all cases, the bees consumed less nicotine than the dose consumed during pre-exposure (Dose table 3.3.1. Extended data table 3.3.1.2 a & e). For example, bees pre-exposed to 25 μM nicotine consumed on average 1.21 μg/bee/day during the pre-exposure period, but during the choice period, they chose a dose that was approximately half of that consumed over the pre-exposure period (0.610 μg/bee/day).

Total mortality was not significantly different between control and treatment groups in the unexposed (Kruskal Wallis test of differences, $\chi^2_{(4)} = 0.318$, $p = 0.957$), or pre-exposed bees (Kruskal Wallis test of differences, $\chi^2_{(4)} = 0.839$, $p = 0.933$).

Extended data table 3.3.1.1 | *A. mellifera* statistics for the 3 day pre-exposure and 24 h two-way choice data for nicotine in 0.5 M sucrose. Data correspond to figure 3.3.1a-c. **(a)** RM-GLM for the total consumed across the 3 day pre-exposure and 24 h two-way choice period. n = 0 μM (24), 25 μM (25), 100 μM (25), 250 μM (25), 500 μM (25). **(b)** GzLM for the indexed 24 h two-way choice day for unexposed and pre-exposed honeybees. n for unexposed bees choice: 0 μM (20), 25 μM (20), 100 μM (20), 250 μM (20), 500 μM (20), n for pre-exposure choice data: 0 μM (24), 25 μM (25), 100 μM (25), 250 μM (25), 500 μM (25). **(c)** GzLM for the total consumed during the 24 h two-way choice period for unexposed and pre-exposed honeybees and one-way ANOVAs for each treatment.

(a)†	Time: $F_{(2.57, 306)} = 232$, $\eta_p^2 = 0.661$, $p = <0.001$ Concentration: $F_{(4, 119)} = 4.10$, $\eta_p^2 = 0.121$, $p = 0.004$ Time x Concentration: $F_{(10.3, 306)} = 2.69$, $\eta_p^2 = 0.083$, $p = 0.003$ *Post hoc* comparisons between time (LSD): All pairwise comparisons $p = <0.001$, with the exception of day 3 vs day 4 where $p = 0.008$ *Post hoc* comparisons between treatments (LSD): 0 μM vs 25 μM $p = 0.02$, 0 μM vs 100 μM $p = <0.001$, 0 μM vs 250 μM $p = <0.001$, 0 μM vs 500 μM $p = 0.003$. *Post hoc* comparisons between Time x Concentration (Bonferroni): Day 1: 0 μM vs 250 μM $p = 0.046$, 0 μM vs 500 μM $p = 0.013$. Day 2: 0 μM vs 25 μM $p = <0.001$, 0 μM vs 100 μM $p = <0.001$, 0 μM vs 250 μM $p = <0.001$, 0 μM vs 500 μM $p = 0.005$. All other pairwise comparisons $p = >0.05$.
(b)	Treatment: $\chi^2_{(1)} = 0.338$, $p = 0.561$ Concentration: $\chi^2_{(4)} = 21.7$, $p = <0.001$ Treatment x Concentration: $\chi^2_{(4)} = 5.81$, $p = 0.214$ *Post hoc* comparisons between concentrations (LSD): 0 μM vs 25 μM $p = 0.184$, 0 μM vs 100 μM $p = 0.963$, 0 μM vs 250 μM $p = 0.049$, 0 μM vs 500 μM $p = 0.006$. One sample T-tests against 0 (Holm-Bonferroni): 0 μM $t_{(19)} = 0.654$ $p = 1.000$, 25 μM $t_{(19)} = -2.47$ $p = 1.000$, 100 μM $t_{(19)} = -1.71$ $p = 0.624$, 250 μM $t_{(19)} = -4.75$. $p = 0.010$, 500 μM $t_{(19)} = -6.20$ $p = 0.010$, 3 day pre-exposure: 0 μM $t_{(23)} = -5.73$ $p = 1.000$, 25 μM $t_{(24)} = 1.63$ $p = 0.624$, 100 μM $t_{(24)} = 0.560$ $p = 1.000$, 250 μM $t_{(24)} = -2.18$ $p = 0.280$, 500 μM $t_{(24)} = -3.01$ $p = 0.048$.
(c)	Treatment: $\chi^2_{(1)} = 196$, $p = <0.001$ Concentration: $\chi^2_{(4)} = 3.73$, $p = 0.444$ Treatment x concentration: $\chi^2_{(4)} = 2.70$, $p = 0.609$ *Post hoc* comparisons between each treatment at each concentration (Bonferroni): All pairwise comparisons $p = <0.001$ One-way ANOVA for unexposed bees: $F_{(4, 95)} = 0.218$ $\eta_p^2 = 0.009$ $p = 0.928$ One-way ANOVA for pre-exposed bees: $F_{(4, 119)} = 1.16$ $\eta_p^2 = 0.037$ $p = 0.333$

† Mauchly's test indicated that the assumption of sphericity has been violated ($\chi^2_{(5)} = 44.6$, $p = <0.001$), therefore Huynh-Feldt corrected tests are reported ($\varepsilon = 0.857$).

Dose Table 3.3.1 | Summary of the average dose consumed by *A. mellifera* following consumption of nicotine in the 24 h two-way choice and pre-exposure periods. Data correspond to figure 3.3.1a-c. Values represent the average nicotine dose consumed per individual honeybee (µg/bee/day). Red indicates the dose where avoidance is observed (See figure 3.3.1b).

[Nicotine] µM	Pre-exposure period dose	Chosen dose	
		Pre-exposed	Unexposed
	µg/bee/day	µg/bee/day	µg/bee/day
25	1.21	0.610	0.737
100	4.61	2.33	2.89
250	11.3	5.39	**6.92**
500	24.0	**9.00**	**13.5**

Extended data table 3.3.1.2 | *A. mellifera* dosage statistics for 24 h and 3 day pre-exposure to nicotine in 0.5 M sucrose. Data correspond to figure 3.3.1 and dose table 3.3.1. Welch's ANOVA for **(a)** the average dose consumed across the 3 day pre-exposure period **(b)** the unexposed 24 h two-way choice data, and **(c)** the 3 day pre-exposed 24 h two-way choice periods. *Post hoc* values are all possible pairwise comparisons between each concentration, Games-Howell adjusted. **(d)** GzLM for the chosen dose during the 24 h two-way choice period between unexposed bees and bees that had been pre-exposed to nicotine for 3 days prior to the choice. *Post hoc* values are comparisons between each treatment schedule at each concentration, LSD adjusted. **(e)** GzLM for the average dose consumed during the 3 day pre-exposure period against the dose chosen following the pre-exposure period. *Post hoc* values are comparisons between the pre-exposure period and the 24 h two-way choice period for each concentration, LSD adjusted.

(a) $F_{(3, 42.2)} = 1260$ $\eta_p^2 = 0.969$ $p = <0.001$. All *post hoc* value comparisons $p = <0.001$.

(b) $F_{(3, 42.2)} = 1470$ $\eta_p^2 = 0.977$ $p = <0.001$. All *post hoc* value comparisons $p = <0.001$.

(c) $F_{(3, 42.7)} = 172$ $\eta_p^2 = 0.780$ $p = <0.001$. All *post hoc* value comparisons $p = <0.001$.

(d) Concentration: $\chi^2_{(3)} = 3050$ $p = <0.001$

Treatment: $\chi^2_{(1)} = 48.7$ $p = <0.001$

Concentration x treatment: $\chi^2_{(3)} = 5.03$ $p = 0.170$

Post hoc comparisons between treatments at each concentration (LSD):

25 µM $p = <0.001$, 100 µM $p = <0.001$, 250 µM $p = <0.001$, 500 µM $p = <0.001$.

(e) Concentration: $\chi^2_{(3)} = 3970$ $p = <0.001$

Treatment: $\chi^2_{(1)} = 525$ $p = <0.001$

Concentration x treatment: $\chi^2_{(3)} = 13.9$ $p = 0.003$

Post hoc comparisons between treatments at each concentration (LSD):

25 µM $p = <0.001$, 100 µM $p = <0.001$, 250 µM $p = <0.001$, 500 µM $p = <0.001$.

3.3.2 The effect of increasing the sucrose concentration to 1.0 M on the honeybee's preference for nicotine

Honeybees pre-exposed to nicotine in a 1.0 M sucrose solution for a period of 3 days varied their total consumption across time as a function of nicotine concentration (Fig. 3.3.2a. RM-GLM, time x concentration, $F_{(5.14,\ 185)} = 4.64$, $\eta_p^2 = 0.661$, $p = < 0.001$). Honeybees pre-exposed to 25 μM nicotine consumed on average 14 μl more solution on the choice day in comparison to control (0 μM) (*Post hoc* Bonferroni $p = <0.001$), and 100 μM nicotine (*Post hoc* Bonferroni $p = 0.035$). Total consumption varied across time in bees pre-exposed to nicotine for a period of 5 days (Fig. 3.3.2b. RM-GLM, time $F_{(2.71,\ 147)} = 6.21$, $\eta_p^2 = 0.103$, $p = < 0.001$), however, the inclusion of nicotine did not affect the total volume consumed over the whole experiment (Extended data table 3.3.2.1b).

The data for the choice test show that there was a significant effect of treatment on choice behaviour (Fig. 3.3.2c. GzLM, treatment, $\chi^2_{(2)} = 0.690$, $p = 0.032$). *Post hoc* analysis revealed that the 3 day pre-exposure treatment was significantly different from the unexposed treatment, but not the 5 day pre-exposure treatment (*Post hoc* LSD, $p = 0.011$ and 0.479, respectively). Despite this, no overall preference was observed for either 25 μM or 100 μM nicotine (Fig. 3.3.2c. Extended data table 3.3.2.1c).

Honeybees in both the 3 day and 5 day pre-exposed treatment groups consumed a significantly lower total volume of solution during the choice period than the unexposed treatment group for all nicotine concentrations (Fig. 3.3.2d. GzLM, treatment x concentration: $\chi^2_{(4)} = 12.6$, $p = 0.013$, Extended data table 3.3.2.1d). However, note that honeybees in the unexposed treatment group and the 5 day pre-exposure group did not consume more or less than their specific sucrose-only control group (Fig. 3.3.2d) (Extended data table 3.3.2.1d). In contrast, honeybees that had been pre-exposed to 25 μM nicotine in the 3 day pre-exposed treatment group consumed a significantly greater total volume of solution over the choice period than bees that had been pre-exposed to sucrose alone (Fig. 3.3.2d) (Extended data table 3.3.2.1d).

Honeybees: Three and five day pre-exposure to nicotine in 1.0 M sucrose

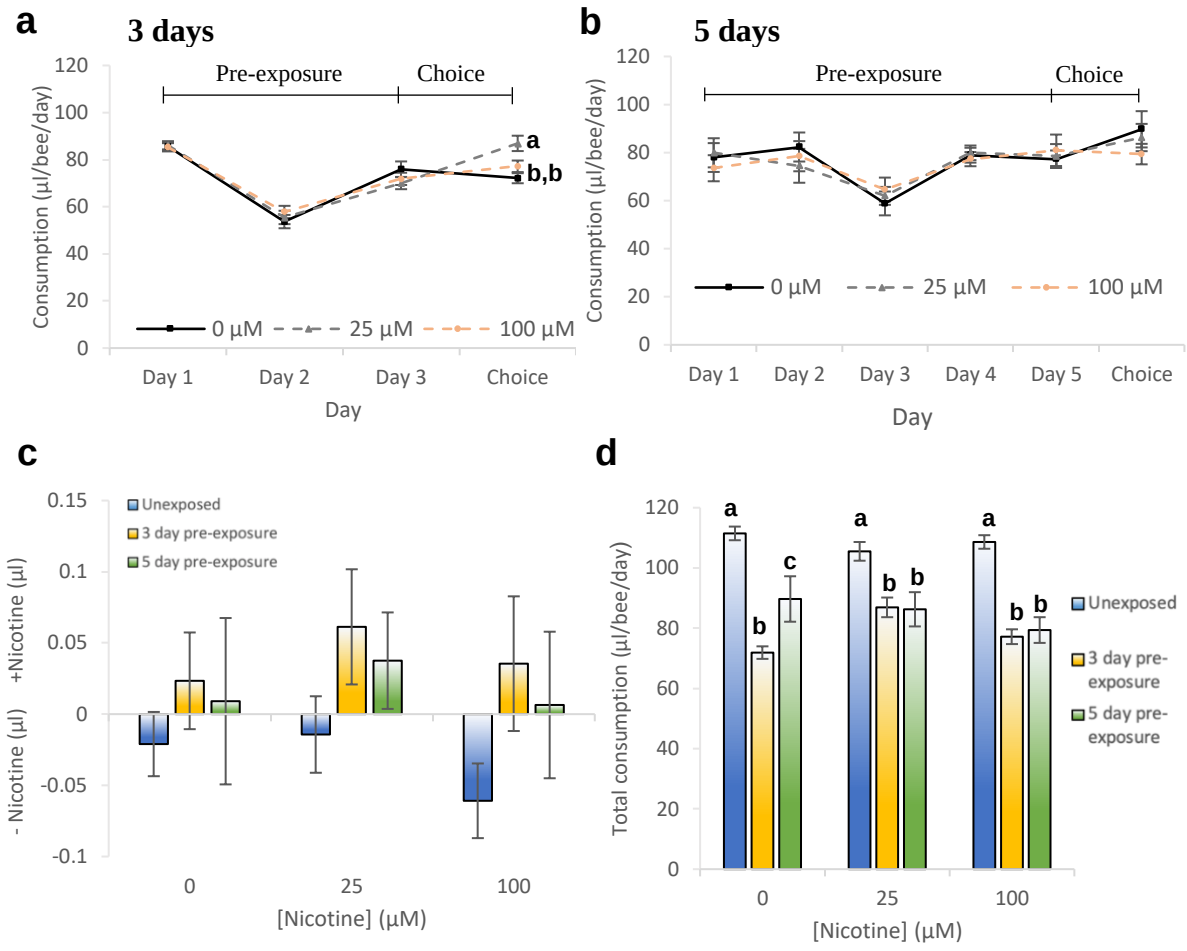

Figure 3.3.2 | Three day or five day pre-exposure to nicotine did not lead to a preference for nicotine when delivered in 1.0 M sucrose. (a) Honeybees total consumption varied as a function of time and nicotine concentration. Honeybees consumed a greater total volume over the choice day when pre-exposed to 25 µM nicotine for 3 days (Extended data table 3.3.2.1a). **(b)** Honeybees varied their total consumption across time (Extended data table 3.3.2.1b); however, the inclusion of nicotine did not alter the total volume consumed over the course of the experiment. **(c)** Pre-exposure to nicotine for 3 or 5 days did not result in a preference for 25 µM or 100 µM nicotine (Extended data table 3.3.2.1c). Data represent the mean difference in the amount consumed over 24 h; positive values indicate a preference for nicotine and negative avoidance of nicotine. **(d)** Honeybees in both pre-exposed treatment groups consumed a significantly lower total volume of solution during the 24 h choice period than the unexposed treatment group for all nicotine concentrations (Extended data table 3.3.2.1c). Both unexposed and 5 day pre-exposed honeybees did not consume a significantly different volume of solution to their respective sucrose-only (0 µM) control groups, whereas honeybees that were pre-exposed to 25 µM nicotine for a period of 3 days were shown to consume a significantly greater total volume of solution over the choice day in comparison to their sucrose-only control. Letters indicate significant differences for *post hoc* comparisons between treatments at each concentration (Bonferroni adjusted). Bars and line graphs indicate mean (±s.e.m.) of consumption (µl) per bee, controlled for by evaporation.

The total dose that the bees consumed over the course of the pre-exposure experiment was also measured (Dose table 3.3.2). The dose of nicotine was 4x greater in the 100 μM pre-exposed bees than bees that consumed 25 μM nicotine (Dose table 3.32. Extended data table 3.3.2.2a-b). For example, honeybees that consumed 25 μM nicotine during the 3 day pre-exposure consumed an average dose of 0.878 μg/bee/day, whereas those that consumed the 100 μM solution consumed an average dose of 3.73 μg/bee/day

During the 24h choice test, honeybees pre-exposed to 100 μM nicotine solutions consumed a lower dose of nicotine than the unexposed honeybees (*Post hoc* Bonferroni: Unexposed 100 μM vs 3 day 100 μM $p = 0.002$, unexposed 100 μM vs 5 day 100 μM $p = 0.018$. Extended data table 3.3.2.2f). As before, pre-exposed honeybees chose a dose over the choice period that was significantly lower than what they consumed during pre-exposure (Dose table 3.3.2. Extended data table 3.3.2.2g-h). For example, bees that consumed 25 μM nicotine consumed 0.878 μg/bee/day during the pre-exposure period, but during the test, they chose to consume a dose of 0.566 μg/bee/day.

Total mortality was not significantly different between the control and treatment groups in the 24 h two-way unexposed choice group (Kruskal Wallis test of differences, $\chi^2_{(2)} = 0.313$, $p = 0.855$), or following 3 days (Kruskal Wallis test of differences, $\chi^2_{(2)} = 4.820$, $p = 0.09$), or 5 days pre-exposure to nicotine (Kruskal Wallis test of differences, $\chi^2_{(2)} = 0.893$, $p = 0.640$).

Extended data table 3.3.2.1 | *A. mellifera* statistics for 3 day and 5 day pre-exposure and 24 h two-way choice data for nicotine in 1.0 M sucrose. Data correspond to figure 3.3.2a-c. **(a)** RM-GLM for the total consumed across the 3 day pre-exposure and 24 h two-way choice period. n = 0 μM (25), 25 μM (25), 100 μM (25). **(b)** RM-GLM for the total consumed across the 5 day pre-exposure and 24 h two-way choice period. n = 0 μM (20), 25 μM (19), 100 μM (18). **(c)** GzLM for the indexed 24 h two-way choice day for unexposed and pre-exposed honeybees. n for unexposed 24 h data: 0 μM (20), 25 μM (20), 100 μM (20). n for 3 day pre-exposure: 0 μM (25), 25 μM (25), 100 μM (25). n for 5 day pre-exposure: 0 μM (20), 25 μM (19), 100 μM (19). **(d)** GzLM for the total consumed during the 24 h two-way choice period for unexposed and pre-exposed honeybees and one-way ANOVAs for each treatment.

(a)†	Time: $F_{(2.57, 185)} = 111$, $\eta_p^2 = 0.606$, $p = <0.001$ Concentration: $F_{(2,72)} = 0.634$, $\eta_p^2 = 0.017$, $p = 0.533$ Time x Concentration: $F_{(5.14, 185)} = 4.64$, $\eta_p^2 = 0.114$, $p = <0.001$ *Post hoc* comparisons between time (LSD): All pairwise comparisons $p = <0.001$ *Post hoc* comparisons between treatments at each time point (Bonferroni): Choice day: 0 μM vs 25 μM $p = <0.001$, 25 μM vs 100 μM $p = 0.035$. All other comparisons $p = >0.05$
(b) ††	Time: $F_{(2.71, 147)} = 6.21$, $\eta_p^2 = 0.103$, $p = <0.001$ Concentration: $F_{(2,54)} = 0.217$, $\eta_p^2 = 0.008$, $p = 0.806$ Time x Concentration: $F_{(5.43, 147)} = 0.422$, $\eta_p^2 = 0.015$, $p = 0.847$ *Post hoc* comparisons between time (Bonferroni): Day 1 vs day 3 $p = 0.041$, day 3 vs day 4 $p = <0.001$, day 3 vs day 5 $p = <0.001$, day 3 vs choice day $p = <0.001$. All other comparisons $p = >0.05$.
(c)	Treatment: $\chi^2_{(2)} = 0.690$, $p = 0.032$ Concentration: $\chi^2_{(2)} = 1.38$, $p = 0.503$ Treatment x Concentration: $\chi^2_{(4)} = 0.606$, $p = 0.962$ *Post hoc* comparisons between treatments (LSD): Unexposed vs 3 day $p = 0.011$, unexposed vs 5 day $p = 0.103$, 3 day vs 5 day $p = 0.479$ One sample T-tests against zero (Holm-Bonferroni adjusted): Unexposed 24 h two-way choice: 0 μM $t_{(29)} = -0.932$ $p = 1.000$, 25 μM $t_{(29)} = -0.533$ $p = 1.000$, 100 μM $t_{(28)} = -2.32$ $p = 0.252$ One sample T-tests against zero for unexposed 3 day 24 h two-way choice: 0 μM $t_{(24)} = 0.689$ $p = 1.000$, 25 μM $t_{(24)} = 1.52$ $p = 1.000$, 100 μM $t_{(24)} = 0.750$ $p = 1.000$. One sample T-tests against zero for unexposed 5 day 24 h two-way choice: 0 μM $t_{(19)} = 0.155$ $p = 1.000$, 25 μM $t_{(18)} = 1.11$ $p = 1.000$, 100 μM $t_{(18)} = 0.125$ $p = 1.000$
	Treatment: $\chi^2_{(2)} = 131$, $p = <0.001$ Concentration: $\chi^2_{(2)} = 2.35$, $p = 0.309$ Treatment x concentration: $\chi^2_{(4)} = 12.6$, $p = 0.013$ *Post hoc* comparisons between treatments (Bonferroni): Unexposed vs 3 day $p = <0.001$, unexposed vs 5 day $p = <0.001$, 3 day vs 5 day $p = 0.116$

(d)

Post hoc comparisons between treatments at each concentration (LSD):

0 μM: Unexposed vs 3 day p = <0.001, unexposed vs 5 day p = <0.001, 3 day vs 5 day p = 0.030. 25 μM: Unexposed vs 3 day p = 0.003, unexposed vs 5 day p = 0.007, 3 day vs 5 day p = 1.000. 100 μM: Unexposed vs 3 day p = <0.001, unexposed vs 5 day p = <0.001, 3 day vs 5 day p = 1.000

One-way ANOVA for unexposed bees: $F_{(2, 86)} = 1.35$ $\eta_p^2 = 0.300$ $p = 0.264$

One-way ANOVA for 3 day pre-exposed bees: $F_{(2, 72)} = 8.01$ $\eta_p^2 = 0.182$ p = <0.001.

Post hoc (Bonferroni): 0 μM vs 25 μM p = <0.001, 0 μM vs 100 μM p = 0.548, 25 μM vs 100 μM p = 0.035

One-way ANOVA for 5 day pre-exposed bees: $F_{(2, 54)} = 0.731$ $\eta_p^2 = 0.026$ $p = 0.486$.

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† Mauchly's test indicated that the assumption of sphericity has been violated ($\chi^2_{(5)} = 29.5$, p = <0.001), therefore Huynh-Feldt corrected tests are reported (ε = 0.803). n = 0 μM (25), 25 μM (25), 100 μM (25).

†† Mauchly's test indicated that the assumption of sphericity has been violated ($\chi^2_{(5)} = 29.5$, p = <0.001), therefore Greenhouse-Geisser corrected tests are reported (ε = 0.543). n = 0 μM (20), 25 μM (19), 100 μM (18).

Dose table 3.3.2 | Summary of the average dose consumed by *A. mellifera* following consumption of nicotine in the 24 h two-way choice periods and during the pre-exposure period. Data correspond to figure 3.3.2a-c. Values represent the average nicotine dose consumed per individual honeybee (μg/bee/day).

[Nicotine] μM & treatment	Pre-exposure period	Chosen dose
	μg/bee/day	μg/bee/day
Unexposed 25	-	0.645
Unexposed 100	-	2.55
3 day 25 pre-exposed	0.878	0.566
3 day pre-exposed 100	3.57	1.98
5 day pre-exposed 25	0.933	0.551
5 day pre-exposed 100	3.73	2.03

Extended data table 3.3.2.2 | *A. mellifera* dosage statistics for 24 h and 3 or 5 day pre-exposure to nicotine in 1.0 M sucrose. Data correspond to figure 3.3.2a-c and dose table 3.3.2. Welch's ANOVA for **(a)** the average dose consumed across the 3 day pre-exposure period **(b)** the average dose consumed across the 5 day pre-exposure period **(c)** Dose chosen during the unexposed 24 h choice **(d)** Dose chosen during the 24 h choice by 3 day pre-exposed bees **(e)** Dose chosen during the 24 h choice by 5 day pre-exposed bees. *Post hoc* values are all possible pairwise comparisons between each concentration, Games-Howell adjusted. **(f)** GzLM for the chosen dose during the 24 h two-way choice period between unexposed bees and bees that had been pre-exposed to nicotine for 3 or 5 days prior to the choice **(g)** GzLM for average dose consumed during the 3 day pre-exposure period, and the dose chosen by pre-exposed bees during the choice test **(h)** GzLM for average dose consumed during the 5 day pre-exposure period, and the dose chosen by pre-exposed bees during the choice test.

(a) $F_{(1, 48)} = 837$, $\eta_p^2 = 0.946$ $p = <0.001$. All *post hoc* value comparisons $p = <0.001$.

(b) $F_{(1, 35)} = 822$, $\eta_p^2 = 0.959$ $p = <0.001$. All *post hoc* value comparisons $p = <0.001$.

(c) $F_{(1, 57)} = 371$, $\eta_p^2 = 0.867$ $p = <0.001$. All *post hoc* value comparisons $p = <0.001$.

(d) $F_{(1, 48)} = 194$, $\eta_p^2 = 0.801$ $p = <0.001$. All *post hoc* value comparisons $p = <0.001$.

(e) $F_{(1, 35)} = 95.3$, $\eta_p^2 = 0.726$ $p = <0.001$. All *post hoc* value comparisons $p = <0.001$.

(f) Concentration: $\chi^2_{(1)} = 1070$ $p = <0.001$

Treatment: $\chi^2_{(2)} = 22.5$ $p = <0.001$

Concentration x treatment: $\chi^2_{(2)} = 1.79$ $p = 0.409$

Post hoc comparisons between treatments at each concentration (Bonferroni): Unexposed 25 µM vs 3 day 25 µM $p = 0.669$, unexposed 25 µM vs 5 day 25 µM $p = 0.335$, 3 day 25 µM vs 5 day 25 µM $p = 1.000$, unexposed 100 µM vs 3 day 100 µM $p = 0.002$, unexposed 100 µM vs 5 day 100 µM $p = 0.018$, 3 day 100 µM vs 5 day 100 µM $p = 1.000$.

(g) Concentration: $\chi^2_{(1)} = 1300$ $p = <0.001$

Treatment: $\chi^2_{(1)} = 195$ $p = <0.001$

Concentration x treatment: $\chi^2_{(1)} = 4.07$ $p = 0.044$

Post hoc comparisons between the average dose consumed during the pre-exposure period and the subsequent chosen dose: 25 µM $p = <0.001$, 100 µM $p = <0.001$.

(h) Concentration: $\chi^2_{(1)} = 704$ $p = <0.001$

Treatment: $\chi^2_{(1)} = 125$ $p = <0.001$

Concentration x treatment: $\chi^2_{(1)} = 0.625$ $p = 0.429$

Post hoc comparisons between the average dose consumed during the pre-exposure period and the subsequent chosen dose: 25 µM $p = <0.001$, 100 µM $p = <0.001$.

3.3.3 The effect of increasing the sucrose concentration to 1.5 M on the honeybee's preference for nicotine

Given that no preference was observed for nicotine when delivered in 1.0 M sucrose, the experiment was repeated with a final sucrose concentration of 1.5 M. As before, honeybees were pre-exposed to either 25 μM or 100 μM nicotine in 1.5 M sucrose for a period of 3 days, before being presented with a 24 h two-way choice between nicotine or 1.5 M sucrose alone.

As seen in the previous experiments, pre-exposure to nicotine affected the amount of solution consumed over the time period (Fig. 3.3.3a. GLM, main effect of Time: $F_{(3, 171)} = 130$, $\eta_p^2 = 0.695$, $p = <0.001$. Extended data table 3.3.3.1a). There was a marginal effect of nicotine concentration (GLM Concentration: $F_{(2,57)} = 2.89$, $\eta_p^2 = 0.092$, $p = 0.064$), with honeybees that were pre-exposed to 100 μM nicotine consuming on average a lower total volume of solution than bees that consumed sucrose alone (Extended data table 3.3.3.1a). In general, the bees consumed as much as ~70 μl on the first day, consumed less on the second day (~40 μl), and consumed slightly more (~65 μl) over the final two days.

The data for the 24 h choice test show that there was a significant effect of treatment and concentration on choice behaviour (Fig.3.3.3b) (GLM, Treatment x Concentration: $F_{(2, 108)} = 3.19$, $\eta_p^2 = 0.056$, $p = 0.045$). *Post hoc* analysis revealed that bees that had been pre-exposed to 25 μM nicotine for a period of 3 days consumed a significantly greater volume of nicotine in comparison to unexposed bees during the 24 h choice period (Extended data table. 3.3.3.1b). Despite this, no overall preference was observed for either 25 μM or 100 μM nicotine (Fig. 3.3.3b. Extended data table. 3.3.3.1b).

As observed previously, honeybees consumed significantly less total volume over the 24 h two-way choice period when they had been pre-exposed to nicotine (Fig 3.3.3c) (GLM, main effect of Treatment: $F_{(1, 108)} = 63.8$, $\eta_p^2 = 0.371$, $p = <0.001$. Extended data table. 3.3.3.1c). However, neither the pre-exposed bees nor the unexposed bees ate significantly less than their respective sucrose-only controls (Fig 3.3.3c. Extended data table. 3.3.3.1c)

Honeybees: 3 day pre-exposure to nicotine in 1.5 M sucrose

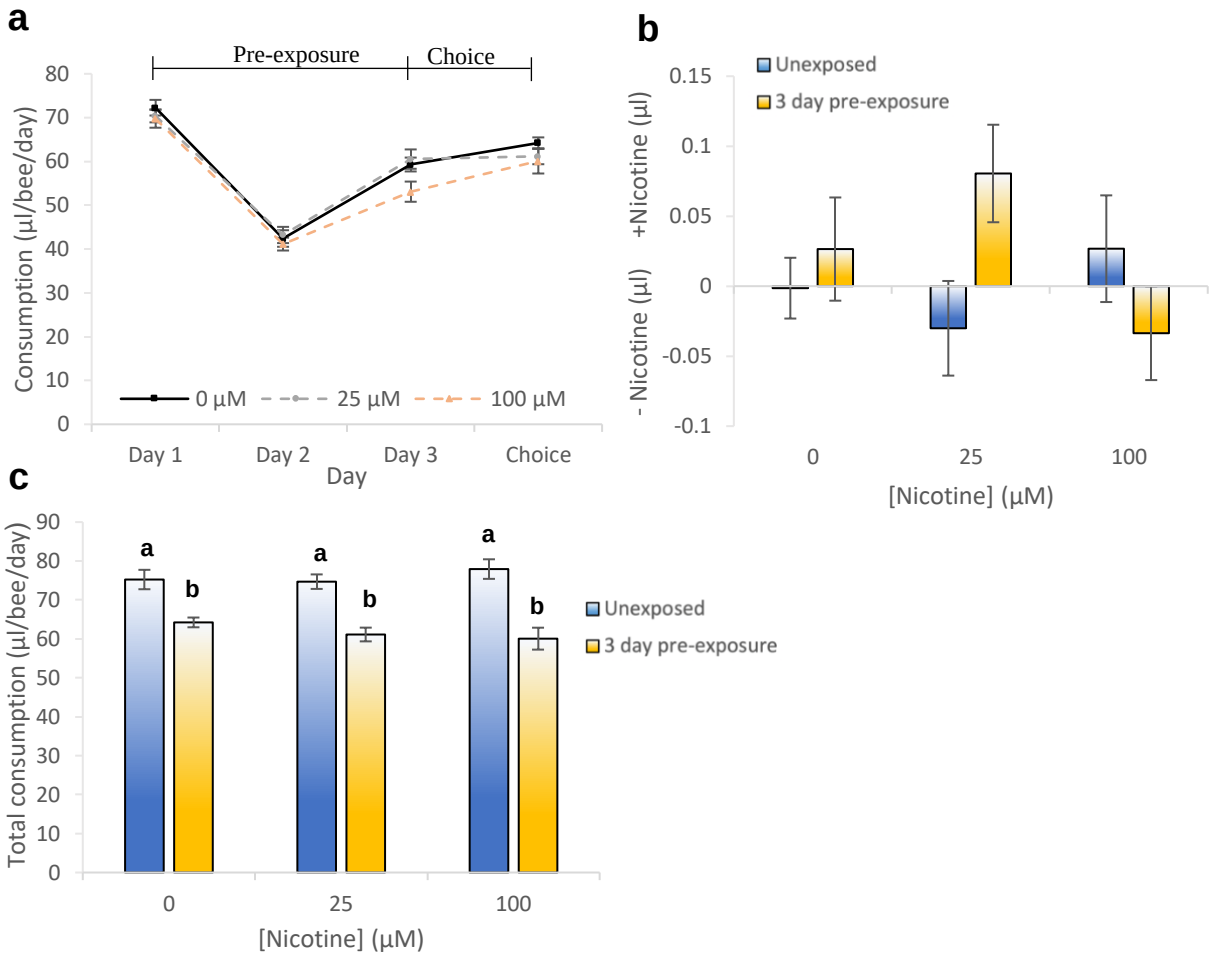

Figure 3.3.3 | Three days pre-exposure to 25 µM or 100 µM nicotine did not lead to a preference for nicotine when delivered in 1.5 M sucrose. (a) Honeybees pre-exposed to nicotine for a period of 3 days varied their total consumption across time (Extended data table. 3.3.3.1a). The inclusion of nicotine resulted in a marginal difference in the total volume consumed across the pre-exposure period (Extended data table. 3.3.3.1c) **(b)** Pre-exposure to nicotine for 3 days did not result in a preference for 25 µM or 100 µM nicotine (Extended data table 3.3.3.1b). Data represent the mean difference in the amount consumed over 24 h; positive values indicate a preference for nicotine and negative avoidance of nicotine. **(c)** Honeybees consumed significantly less total volume of solution over the 24 h two-way choice period when they had been pre-exposed to nicotine opposed to unexposed bees (Extended data table. 3.3.3.1c). However, note that neither the pre-exposed bees nor the unexposed bees consumed significantly less than their respective sucrose-only controls (Extended data table. 3.3.3.1c). Letters indicate significant differences for *post hoc* comparisons between treatments at each concentration (LSD). Bars and line graphs indicate mean (±s.e.m.) of consumption (µl) per bee, controlled for by evaporation.

The total dose that the bees consumed over the course of the experiment was also measured (Dose table 3.3.3). The dose of nicotine among the 100 μM pre-exposed bees was as much as 4x greater than the 25 μM pre-exposed bees (Dose table 3.3.3. Extended data table 3.3.3.2a-b). Pre-exposed honeybees that were presented with 25 μM nicotine consumed an average dose of 0.722 μg/bee/day, whereas those presented with 100 μM solutions consumed an average dose of 2.72 μg/bee/day.

The concentration of nicotine affected whether the bees ate a lower dose of nicotine in the 24 h choice test if they had been pre-exposed (GzLM, Concentration x treatment: $\chi^2_{(1)} = 6.07$ $p = 0.014$. *Post hoc* LSD, p = <0.001 and 1.00 respectively). Honeybees pre-exposed to 25 μM chose to consume a dose of 0.424 μg/bee/day, whereas unexposed honeybees chose a dose of 0.448 μg/bee/day. In contrast, honeybees pre-exposed to 100 μM nicotine chose to consume a dose of 1.47 μg/bee/day, whereas unexposed honeybees chose a dose of 2.00 μg/bee/day. In both cases, honeybees pre-exposed to nicotine ate less nicotine in the choice test than they had during the pre-exposure period (GzLM main effect of treatment, $\chi^2_{(1)} = 174$ $p = <0.001$. Extended data table 3.3.3.2e).

Total mortality was not significantly different between control and treatment groups in the 24 h two-way unexposed (Kruskal Wallis test of differences, $\chi^2_{(2)} = 0.199$, $p = 0.905$), or pre-exposed bees (Kruskal Wallis test of differences, $\chi^2_{(2)} = 0.042$, $p = 0.837$).

Extended data table 3.3.3.1 | *A. mellifera* statistics for 24 h and 3 day pre-exposure to nicotine in 1.5 M sucrose. Data correspond to figure 3.3.3a-c. **(a)** RM-GLM Total consumption across the 3 day pre-exposure and 24 h two-way choice period. n = 0 μM (20), 25 μM (20), 100 μM (20). **(b** GLM, 24 h two-way choice data for unexposed and pre-exposed honeybees. n = unexposed: 0 μM (18), 25 μM (18), 100 μM (18). Pre-exposed bees: n = 0 μM (20), 25 μM (20), 100 μM (20). **(c)** GLM, Total consumption during the 24 h two-way choice period for unexposed and pre-exposed honeybees and one-way ANOVAs for each treatment.

(a)	Time: $F_{(3, 171)} = 130$, $\eta_p^2 = 0.695$, $p = <0.001$ Concentration: $F_{(2,57)} = 2.89$, $\eta_p^2 = 0.092$, $p = 0.064$ Time x Concentration: $F_{(6, 171)} = 0.957$, $\eta_p^2 = 0.032$, $p = 0.456$ *Post hoc* comparisons between time (LSD): All pairwise comparisons $p = <0.001$, with the exception of day 3 vs choice day where $p = 0.004$ *Post hoc* comparisons between concentrations (LSD): 0 μM vs 25 μM $p = 0.638$, 0 μM vs 100 μM $p = 0.026$, 25 μM vs 100 μM $p = 0.076$.
(b)	Treatment: $F_{(1, 108)} = 0.889$, $\eta_p^2 = 0.008$, $p = 0.348$ Concentration: $F_{(2, 108)} = 0.360$, $\eta_p^2 = 0.007$, $p = 0.699$ Treatment x Concentration: $F_{(2, 108)} = 3.19$, $\eta_p^2 = 0.056$, $p = 0.045$ *Post hoc* pairwise comparisons between treatments at each concentration: 0 μM $p = 0.561$, 25 μM $p = 0.023$, 100 μM $p = 0.210$ One sample T-tests against 0 (Holm-Bonferroni): unexposed 0 μM $p = 1.000$, 25 μM $p = 1.000$, 100 μM $p = 1.000$. 3 day pre-exposure: 0 μM $p = 1.000$, 25 μM $p = 0.192$, 100 μM $p = 1.000$.
(c)†	Treatment: $F_{(1, 108)} = 63.8$, $\eta_p 2 = 0.371$, $p = <0.001$ Concentration: $F_{(2, 108)} = 0.391$, $\eta_p^2 = 0.007$, $p = 0.678$ Treatment x Concentration: $F_{(2, 108)} = 1.46$, $\eta_p^2 = 0.026$, $p = 0.237$ *Post hoc* comparisons between treatments at each concentration (LSD): All pairwise comparisons $p = <0.001$. One-way ANOVA for unexposed bees: $F_{(2, 51)} = 0.499$ $\eta_p^2 = 0.019$ $p = 0.610$ One-way ANOVA for pre-exposed bees: $F_{(2, 57)} = 1.40$ $\eta_p^2 = 0.047$ $p = 0.255$

† Data was square-root transformed.

Dose table 3.3.3 | Summary of the average dose consumed by *A. mellifera* following consumption of nicotine in the 24 h two-way choice and pre-exposure periods. Data correspond to figure 3.3.3. Values represent the average nicotine dose consumed per individual honeybee dose (µg/bee/day).

[Nicotine] µM	Pre-exposure period dose	Chosen dose	
		Pre-exposed	Unexposed
	µg/bee/day	µg/bee/day	µg/bee/day
25	0.448	0.423	0.722
100	2.00	1.47	2.72

Extended data table 3.3.3.2 | *A. mellifera* dosage statistics for 24 h and 3 day pre-exposure to nicotine. Data correspond to figure 3.3.3 and dose table 3.3.3. Welch's ANOVA for **(a)** the average dose consumed across the 3 day pre-exposure period **(b)** the unexposed bees 24 h two-way choice data, and **(c)** the 3 day pre-exposed 24 h two-way choice periods. *Post hoc* values are all possible pairwise comparisons between each concentration, Games-Howell adjusted. **(d)** GzLM for the chosen dose during the 24 h two-way choice period between unexposed bees and bees that had been pre-exposed to nicotine for 3 days prior to the choice. **(e)** GzLM for the average dose consumed during the 3 day pre-exposure period against the dose chosen following the pre-exposure period.

(a) $F_{(1, 23.8)} = 1750$ $\eta_p^2 = 0.979$ $p = <0.001$. All *post hoc* value comparisons $p = <0.001$.

(b) $F_{(1, 17.8)} = 218$ $\eta_p^2 = 0.865$ $p = <0.001$. All *post hoc* value comparisons $p = <0.001$.

(c) $F_{(1, 19.8)} = 76.2$ $\eta_p^2 = 0.667$ $p = <0.001$. All *post hoc* value comparisons $p = <0.001$.

(d) Concentration: $\chi^2_{(1)} = 651$ $p = <0.001$

Treatment: $\chi^2_{(1)} = 11.1$ $p = <0.001$

Concentration x treatment: $\chi^2_{(1)} = 6.07$ $p = 0.014$

Post hoc comparisons between treatments at each concentration (LSD):

25 µM $p = 1.000$, 100 µM $p = <0.001$,

(e) Concentration: $\chi^2_{(1)} = 872$, $p = <0.001$

Treatment: $\chi^2_{(1)} = 174$ $p = <0.001$

Concentration x treatment: $\chi^2_{(1)} = 1.18$ $p = 0.277$

Post hoc comparisons between treatments at each concentration (LSD):

25 µM $p = <0.001$, 100 µM $p = <0.001$.

3.3.4 The effect of intermittent pre-exposure on the honeybee's preference for nicotine

The honeybees in the I12 treatment group showed a distinct pattern of total food consumption that depended on the schedule and the concentration of nicotine in food (Fig 3.3.4a Time x Concentration: $F_{(10, 260)} = 2.27$, $\eta_p^2 = 0.080$, $p = 0.015$). Honeybees consumed as much as 78 µl over the initial 12 h period. This decreased to ~20 µl during the following 12 h. The total volume consumed then oscillated over the remaining 2 days for all treatment groups. *Post-hoc* analysis revealed that honeybees in the 25 µM nicotine group consumed significantly more solution than the control at the 24 h time point, but a significantly lower volume at the 36 h time point (Bonferroni $p = 0.018$ and $p = 0.028$, respectively), representing a change in volume of ~7 µl for both time points.

The honeybees in the I48 treatment group showed a distinct pattern of total food consumption that depended on the schedule and the concentration of nicotine in solution (Fig 3.3.4b. RM-GLM, Time x Concentration: $F_{(2.30, 159)} = 0.422$, $\eta_p^2 = 0.080$, $p = 0.037$). In general, bees exposed to nicotine over the course of the experiment consumed less solution (*Post hoc* Bonferroni: 0 µM vs 25 µM $p = 0.006$, 0 µM vs 100 µM $p = 0.022$). For example, at 96 h, nicotine-exposed bees consumed on average ~10 µl less total solution in comparison to control bees.

In contrast to the data for the continuous nicotine pre-exposure in earlier experiments, honeybees displayed a significant preference for solutions containing nicotine in both the I12 and I48 exposure treatments (Fig 3.3.4c). The preference for nicotine was a function of both nicotine treatment and the duration of pre-exposure periods (GzLM, Treatment x Concentration: $\chi^2_{(4)} = 9.51$, $p = 0.049$). Honeybees experiencing the 12 h intermittent schedule (I12) exhibited a preference for 100 µM nicotine (Extended data table 3.3.4.1c), whereas honeybees experiencing the 48 h intermittent schedule (I48) showed a preference for 25 µM nicotine (Extended data table 3.3.4.1c).

Mean total consumption of solution over the 24 h two-way choice period was also assessed (Fig. 3.3.4d). Total consumption was shown to vary as a function of treatment

Honeybees: Intermittent pre-exposure to nicotine in 1.0 M sucrose

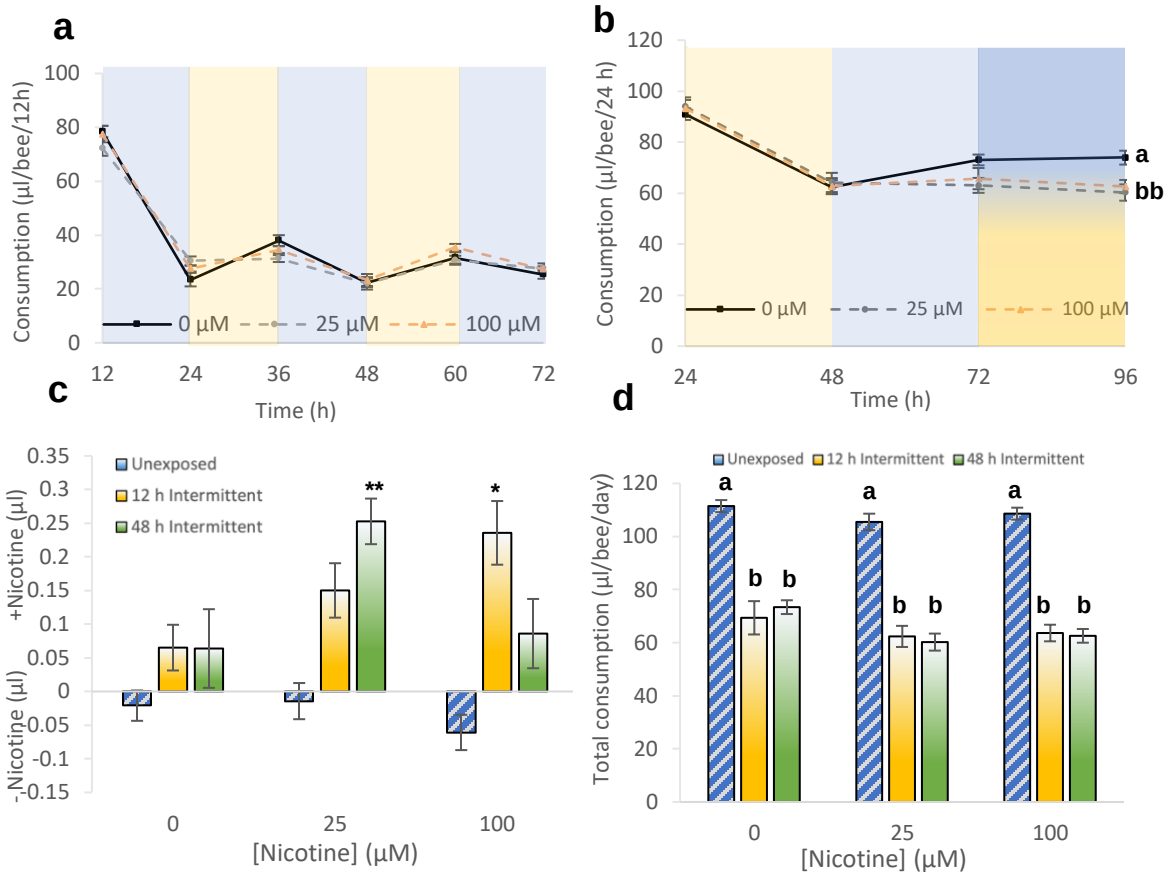

Figure 3.3.4 | Intermittent pre-exposure results in a significant preference for nicotine in the honeybee (a) Total consumption across the pre-exposure period for intermittent 12 (I12) exposed bees. Honeybees varied their total consumption across time; however, in general, the inclusion of nicotine did not alter the total volume consumed (Extended data table 3.3.4.1a). Yellow regions indicate periods of nicotine exposure and blue periods of sucrose exposure. **(b)** Total consumption across the pre-exposure and 24 h two-way choice period for intermittent 48 (I48) exposed bees. Total consumption varied as a function of both time and concentration (Extended data table 3.3.4.1b). Mean total consumption of solution decreased in bees that were exposed to both 25 µM and 100 µM nicotine over the course of the experiment (Extended data table 3.3.4.1b). Yellow regions indicate periods of nicotine exposure and blue periods of sucrose exposure. **(c)** Honeybees that underwent the I12 schedule of pre-exposure exhibited a preference for 100 µM nicotine, whereas bees that underwent the I48 schedule exhibited a preference for 25 µM nicotine (Extended data table 2.7b). No preference was observed in unexposed bees (Extended data table 3.3.4.1b). Data represent the mean difference in the amount consumed over 24 h, positive values indicate a preference for nicotine and negative avoidance of nicotine. (* p <0.05, ** p <0.01). Dashed lines represent data obtained for 1.0 M unexposed honeybees collected in section 3.3.3, used for comparison here. **(d)** Total consumption over the 24 h two-way choice period. Total consumption varied as a function of treatment and concentration (Extended data table 3.3.4.1d). Nicotine-exposed honeybees consumed a lower total volume of solution in comparison to unexposed bees (Extended data table 3.3.4.1d). Letters indicate significant differences for *post hoc* comparisons between treatments at each concentration (Bonferroni adjusted). Bars and line graphs indicate mean (±s.e.m.) of consumption (µl) per bee, controlled for by evaporation. Dashed lines represent data obtained for 1.0 M unexposed honeybees collected in section 3.3.3, used for comparison here.

(GzLM, treatment main effect $\chi^2_{(2)} = 368$ $p = <0.001$), and nicotine concentration (GzLM, concentration main effect $\chi^2_{(2)} = 10.2$, $p = 0.006$). As before, pre-exposed bees ate less solution than unexposed bees (Extended data table 3.3.4.1d). Honeybees that had been pre-exposed to nicotine in the I12 schedule of administration did not consume significantly more or less solution than their specific sucrose-only control group (Extended data table 3.3.4.1d). However, bees that had been pre-exposed to nicotine in the I48 schedule of administration consumed a significantly lower total volume of solution than their specific sucrose-only control group (Extended data table 3.3.4.1d).

As before, the total dose that the bees consumed over the course of the experiment was also measured (Dose table 3.3.4). The dose of nicotine among the pre-exposed bees was as much 7x greater than the lowest nicotine concentration, depending on the treatment (Dose table 3.3.4. Extended data table 3.3.4.2a-b). For example, over the I12 pre-exposure period, honeybees fed with 25 μM nicotine consumed an average dose of 0.577 μg/bee/day (note that the dose consumed over a day is identical in value to that consumed over an individual 12 h period), whereas, honeybees that consumed 100 μM nicotine on the I48 schedule consumed an average dose of 3.85 μg/bee/day.

As before, pre-exposed bees consumed a significantly lower dose during the choice test than unexposed honeybees (Extended data table 3.3.4.2c-d). The nicotine dose consumed during pre-exposure did not affect the dose consumed in the test (25 μM or 100 μM) (Dose table 3.3.4 Extended data table 3.3.4.2c-d). For example, honeybees that consumed 25 μM nicotine on the I12 pre-exposure schedule chose a dose of 0.441 μg/bee/day (in comparison to the average pre-exposure dose of 0.577 μg/bee/day), whereas honeybees that consumed 25 μM on the I48 pre-exposure schedule chose a dose of 0.464 μg/bee/day (in comparison to the average pre-exposure dose of 0.988 μg/bee/day).

Total mortality was not significantly different between control and treatment groups in the unexposed bees (Kruskal Wallis test of differences, $\chi^2_{(2)} = 0.313$, $p = 0.855$), or following the I12 pre-exposure treatment (Kruskal Wallis test of differences, $\chi^2_{(2)} = 1.43$, $p = 0.490$), or following the I48 pre-exposure treatment (Kruskal Wallis test of differences, $\chi^2_{(2)} = 0.071$, $p = 0.965$).

Extended data table 3.3.1.1 | *A. mellifera* statistics for unexposed, I12 (12 h intermittent) and I48 (48 h intermittent) treatments. Data correspond to figure 3.3.4a-c. **(a)** RM-GLM for the total consumed across the I12 treatment. n = 0 μM (19), 25 μM (19), 100 μM (20). **(b)** RM-GLM for the total consumed across the I48 treatment. n = 0 μM (20), 25 μM (19), 100 μM (20). **(c)** GzLM for the indexed 24 h two-way choice day for unexposed, I12, and I48 treatments **(d)** GzLM for the total consumed during the 24 h two-way choice period for unexposed, I12 and I48 treatments, and one-way ANOVAs for each treatment.

(a)†	Time: $F_{(1, 260)} = 262$, $\eta_p^2 = 0.834$, $p = <0.001$ Concentration: $F_{(2,52)} = 0.980$, $\eta_p^2 = 0.036$, $p = 0.382$ Time x Concentration: $F_{(10, 260)} = 2.27$, $\eta_p^2 = 0.080$, $p = 0.015$ *Post hoc* comparisons between time (Bonferroni): 12 h vs all other time points $p = <0.001$. 24 h vs 36 h $p = <0.001$, 24 h vs 48 h $p = 0.006$, 24 h vs 60 h $p = <0.001$, 36 h vs 48 h $p = <0.001$, 36 h vs 72 h $p = <0.001$, 48 h vs 60 h $p = <0.001$, 48 h vs 72 h $p = 0.006$, 60 h vs 72 h $p = <0.001$. All other pairwise comparisons $p = >0.05$. *Post hoc* comparisons across each concentration at each time point (Bonferroni), only significant differences are listed: 24 h: 0 μM vs 25 μM $p = 0.018$. 36 h: 0 μM vs 25 μM $p = 0.028$.
(b) †	Time: $F = 63.3_{(3, 159)}$, $\eta_p^2 = 0.544$, $p = <0.001$ Concentration: $F_{(2,53)} = 2.27$, $\eta_p^2 = 0.079$, $p = 0.113$ Time x Concentration: $F_{(2.30, 159)} = 0.422$, $\eta_p^2 = 0.080$, $p = 0.037$ *Post hoc* comparisons between time (LSD): 24 h vs 48 h $p = <0.001$, 24 h vs 72 h $p = <0.001$, 24 h vs 96 h $p = <0.001$. All other pairwise comparisons $p = >0.05$. *Post hoc* comparisons between each concentration at each time point (Bonferroni), only significant differences are listed: 96 h: 0 μM vs 25 μM $p = 0.006$, 0 μM vs 100 μM $p = 0.022$.
(c)	Treatment: $\chi^2_{(2)} = 28.5$, $p = <0.001$ Concentration: $\chi^2_{(2)} = 5.32$, $p = 0.70$ Treatment x Concentration: $\chi^2_{(4)} = 9.51$, $p = 0.049$ *Post hoc* comparisons between treatments at each concentration (Holm-Bonferroni), only significant values are reported: 25 μM unexposed vs 25 μM I48 $p = 0.003$, 100 μM unexposed vs 100 μM I12 $p = <0.001$. One sample T-tests against zero (Holm-Bonferroni adjusted): Unexposed 24 h two-way choice: 0 μM $t_{(29)} = -0.932$ $p = 1.000$, 25 μM $t_{(29)} = -0.533$ $p = 1.000$, 100 μM $t_{(28)} = -2.32$ $p = 0.196$. One sample T-tests against zero for I12 day 24 h two-way choice: 0 μM $t_{(18)} = 0.996$ $p = 1.000$, 25 μM $t_{(18)} = 2.27$ $p = 0.216$, 100 μM $t_{(19)} = 3.930$ $p = <0.008$ One sample T-tests against zero for I48 day 24 h two-way choice: 0 μM $t_{(19)} = 0.913$ $p = 1.000$, 25 μM $t_{(17)} = 3.45$ $p = 0.024$, 100 μM $t_{(19)} = 1.24$ $p = 1.000$

Treatment: $\chi^2_{(2)} = 368$ $p = <0.001$

Concentration: $\chi^2_{(2)} = 10.2$, $p = 0.006$

Treatment x Concentration: $\chi^2_{(4)} = 1.19$, $p = 0.880$

Post hoc comparisons between concentrations (Holm-Bonferroni):

0 µM vs 25 µM $p = <0.001$, 0 µM vs 100 µM $p = 0.015$, 25 µM vs 100 µM $p = 0.383$

Post hoc comparisons between treatments (LSD):

Unexposed vs I12 $p = <0.001$, unexposed vs I48 $p = <0.001$, I12 vs I48 $p = 1.000$

(d) *Post hoc* comparisons between treatments at each concentration (Holm-Bonferroni):

0 µM: unexposed vs I12 $p = <0.001$, unexposed vs I48 day $p = <0.001$, I12 vs I48 $p = 1.000$.

25 µM: unexposed vs I12 $p = <0.001$, unexposed vs I48 $p = <0.001$, I12 vs I48 $p = 1.000$.

100 µM: unexposed vs I12 $p = <0.001$, unexposed vs I48 $p = <0.001$, I12 vs I48 $p = 1.000$

One-way ANOVA for unexposed bees: $F_{(2, 51)} = 0.499$ $\eta_p^2 = 0.019$ $p = 0.610$

One-way ANOVA for I12 bees: $F_{(2, 54)} = 0.615$ $\eta_p^2 = 0.022$ $p = 0.544$

One-way ANOVA for I48 bees: $F_{(2, 56)} = 6.313$ $\eta_p^2 = 0.184$ $p = 0.003$

Post hoc (Bonferroni): 0 µM vs 25 µM $p = 0.005$, 0 µM vs 100 µM $p = 0.024$, 25 µM vs 100 µM $p = 1.000$,

† Data was square-root transformed.

Dose table 3.3.4 | Summary of the average dose consumed by *A. mellifera* following consumption of nicotine in the 24 h two-way choice and intermittent pre-exposure periods. Data correspond to figure 3.3.4. Values represent the average nicotine dose (µg/bee/12 h or µg/bee/24 h) per individual honeybee over each 12 h period for the I12 treatment and the average dose consumed over the initial 48 h for the I48 treatment. Green indicates a preference for nicotine (See figure 3.3.4).

[Nicotine] µM & treatment	**Average dose across the pre-exposure period**	**Chosen dose**
	I12: µg/bee/12 h I48: µg/bee/24 h	**µg/bee/day**
Unexposed 25	-	0.645
Unexposed 100	-	2.55
I12 25	0.557	0.441
I12 100	2.45	**1.92**
I48 25	0.988	**0.464**
I48 100	3.85	1.67

Extended data table 3.3.4.2 | *A. mellifera* dosage statistics for unexposed and I12 and I48 pre-exposed honeybees. Data correspond to figure 3.3.4 and dose table 3.3.4. **(a)** GzLM comparing the pre-exposed dose consumed across the 25 μM I12 and I48 treatments against the I12 and I48 chosen dose for 25 μM. **(b)** GzLM comparing the pre-exposed dose consumed across the 100 μM I12 and I48 treatments against the I12 and I48 chosen dose for 100 μM. **(c)** One-Way ANOVA for the 25 μM chosen dose between the unexposed 24 h two-way choice and the chosen dose in the I12 and I48 treatments. **(d)** One-Way ANOVA for the 100 μM chosen dose between the unexposed 24 h two-way choice and the chosen dose in the I12 and I48 treatments.

(a) Time: $\chi^2_{(1)} = 123$ $p = <0.001$

Treatment: $\chi^2_{(1)} = 62.0$ $p = <0.001$

Time x Treatment: $\chi^2_{(1)} = 50.1$ $p = <0.001$

Post hoc comparisons between treatments at each concentration (LSD):

I12 pre-exposure vs I12 choice $p = 0.004$, I48 pre-exposure vs I48 choice $p = <0.001$, I12 pre-exposure vs I48 pre-exposure, $p = <0.001$, I12 choice vs I48 choice $p = 0.568$

(b) Time: $\chi^2_{(1)} = 190$ $p = <0.001$

Treatment: $\chi^2_{(1)} = 33.9$ $p = <0.001$

Time x Treatment: $\chi^2_{(1)} = 70.1$ $p = <0.001$

Post hoc comparisons between treatments at each concentration (LSD):

I12 pre-exposure vs I12 choice $p = <0.001$, I48 pre-exposure vs I48 choice $p = <0.001$, I12 pre-exposure vs I48 pre-exposure, $p = <0.001$, I12 choice vs I48 choice $p = 0.067$.

(c) $F_{(2, 65)} = 17.3$ $\eta_p^2 = 0.979$ $p = <0.001$. *Post hoc* Bonferroni. unexposed vs I12 $p = <0.001$, unexposed vs I48 $p = <0.001$, I12 vs I48 $p = 1.000$

†(d) $F_{(2, 66)} = 21.4$ $\eta_p^2 = 0.979$ $p = <0.001$. *Post hoc* Bonferroni. unexposed vs I12 $p = <0.001$, unexposed vs I48 $p = <0.001$, I12 vs I48 $p = 0.214$.

†Data was square-root transformed

3.4 Discussion

The results indicate that the schedule of drug reinforcement has a significant effect on whether a preference for nicotine is acquired by adult worker honeybees. Bees chronically pre-exposed to nicotine did not exhibit a preference for the alkaloid. In contrast, when honeybees experienced intermittent pre-exposure to nicotine over a 3 day period, they showed a preference for food containing the compound.

3.4.1 Honeybees vary their total consumption across time

Across experiments, pre-exposed honeybees typically consumed a greater total volume of food on the first day, followed by a decline in consumption on the second day, after which the total consumption remained approximately equal during the final two days. The trend observed in the total volume consumed is typical in caged experiments in honeybees (Paris et al. 2017). This is likely due to the starvation period the bees undergo during the collection period, where bees may be housed in collection vials for 1-3 h prior to being placed into the feeding cages, and starvation is known to increase the honeybees consumption of food relative to non-starved bees (Desmedt et al. 2016). It is important to note that the time taken to collect honeybees can vary widely on a particular day, not only depending upon the number of bees required for experimentation but in large part due to weather conditions. Fluctuations in daily temperatures (Vicens and Bosch 2000), solar radiation (Burrill and Dietz, 1981), and wind speed (Hennessy et al. 2020) can all alter the bees willingness to forage, and therefore the ability to collect the bees on a given day. As such, the total volume consumed on the first day may vary across experiments as an increase in collection time results in an increase in the starvation period prior to experimentation. This is a caveat that holds for both pre-exposed and unexposed bees in this study. In addition, the collection and caging procedures are likely to be moderately stressful to bees, and stress is known to increase the metabolic rate of insects (Burggren et al. 2017). Indeed, the metabolic rate of harnessed honeybees is high immediately following harnessing but decreases over the course of the day (Kazlauskas et al. 2016). This decline in metabolic rate over time is thought to reflect a reduction in stress as the honeybee becomes habituated to the test environment (Kazlauskas et al. 2016). Thus, a combination of starvation and stress endured through collection could account for the increase in the total volume consumed on the first day of experimentation in pre-exposed honeybees.

Although increased consumption immediately following collection is common in the honeybee literature, periods of experimental habituation are not. Future studies may therefore allow for a period of habitation to the experimental environment prior to experimentation to normalise for the differences in collection time and the stress placed on the bee following collection. At present, it is not clear what could account for the decline in consumption on the second day of experimentation, followed by a gradual increase in consumption thereafter. One possible explanation is that stressed honeybees may over-eat on the first day of experimentation and thus may require less food the following day; however, this has not been empirically tested.

Although not directly assessed in this experiment, the results indicate that the pre-exposed honeybees consumed different volumes on the first day, depending on the sucrose concentration used. For instance, control bees that were provided with a 0.5 M sucrose solution consumed 143 µl on the first day. In contrast, control bees that were provided with a 1.0 M or 1.5 M sucrose solution consumed 85 µl and 72 µl during their first day, respectively. This result is in line with the honeybees regulating their intake of carbohydrates to reach a specific optima (Simpson and Raubenheimer, 2012). Indeed, previous work on honeybees has shown that adult worker honeybees strongly regulate their intake of carbohydrates (Paoli et al. 2014). Furthermore, bumblebees are known to consume approximately twice as much of a 0.25 M sucrose solution as a 0.5 M solution in order to reach their carbohydrate needs (Stabler et al. 2015). As such, it was expected that honeybees would consume a greater total volume of a 0.5 M sucrose solution, opposed to a 1.0 M or 1.5 M sucrose solution, as observed. In addition, honeybees were expected to consume a greater total volume of 1.0 M sucrose in comparison to bees provided with a 1.5 M solution; however, this was not the case, and a difference of only 13 µl was observed between these sucrose concentrations. Although the total volume consumed by the bees presented with 1.5 M sucrose was greater than expected, this result may be explained by the timing of the respective experiments; bees tested with the 0.5 M and 1.0 M sucrose solutions were collected in peak summer (June-August), whereas those tested with the 1.5 M sucrose solution were collected in early autumn (September).

Honeybees are known to exhibit large seasonal variability in their physiology (Münch and Amdam, 2010). For instance, honeybees in the peak summer months (June-

August) have higher stores of macronutrients in comparison to bees in early autumn (September-October) (Shehata et al. 2015). In addition, early autumn bees express vitellogenin, a hormone responsible for worker food-choice behaviour (Fennern and Havukainen, 2011), at a significantly lower level than summer bees (Steinmann et al. 2015). Vitellogenin knock-down studies in honeybees indicate that a reduction in vitellogenin results in an increase in carbohydrate consumption (Nelson et al. 2007). As such, the bees tested with the 1.5 M sucrose solution likely had reduced macronutrient stores and vitellogenin expression in comparison to the bees collected between June-August. This could account for increased carbohydrate consumption in the 1.5 M experiments, and hence a greater total volume of food consumed to that expected.

3.4.2 Nicotine affects the total volume of food consumed by honeybees only when bees have been pre-exposed to nicotine

Honeybees were shown to decrease the total volume of food they consumed over the pre-exposure period in three of the pre-exposure protocols. In the 0.5 M sucrose 3 day pre-exposure treatment, honeybees that were provided with 250 μM and 500 μM nicotine in 0.5 M sucrose consumed significantly less total food than their sucrose-only control on the first day of the pre-exposure period. On the second day, all nicotine treatments (25, 100, 250, and 500 μM nicotine) were shown to consume less total food than bees that were provided with sucrose alone. However, this effect was no longer apparent on the third day of pre-exposure or over the choice day, indicating that any effect nicotine had on suppressing feeding in the honeybee was abolished over the remaining two days of the experiment. The second instance of nicotine suppressing feeding was observed in honeybees that had been fed 100 μM nicotine in 1.5 M sucrose (note marginal result). Here, honeybees were shown to reduce the total volume of food they consumed relative to honeybees that consumed sucrose alone. However, as before, any effect nicotine had on suppressing feeding was no longer apparent over the choice period. The final instance was seen in honeybees that consumed nicotine on the I12 schedule of administration. Here, honeybees that were pre-exposed to 25 μM nicotine intermittently decreased the total volume of food that they consumed following their second period of nicotine exposure (i.e. at the 36 h time point) relative to their sucrose-only control group. Again, this result was shown to be only transient, as honeybees that were pre-exposed to 25 μM nicotine

intermittently consumed approximately equal volumes of food to their sucrose control group for the remainder of the experiment. Collectively, this suggests that honeybees may decrease the total volume of food that they consume when they are forced to consume nicotine in either a chronic or intermittent fashion over an extended period of time; however, this result does not appear to be due to the dose of nicotine consumed, or the sucrose molarity that nicotine is administered in. For example, honeybees that were pre-exposed to 25 μM nicotine in 0.5 M sucrose decreased the total volume of food they consumed relative to their sucrose control group over the pre-exposure period, whilst consuming an average dose of 1.21 μg/bee/day. In contrast, honeybees that were pre-exposed to 25 μM and 100 μM nicotine in 1.0 M sucrose for 3 days did not decrease the total volume of food they consumed relative to their sucrose control over the pre-exposure period, despite consuming an approximately equal dose of nicotine over the pre-exposure period (25 μM dose = 0.878 μg/bee/day, 100 μM dose = 3.57 μg/bee/day). This indicates that honeybees that were pre-exposed to 25 μM nicotine in 0.5 M sucrose did not reduce the total volume of food that they ate over the pre-exposure period due to the dose of nicotine consumed, i.e. if a nicotine dose of 1.21 μg/bee/day is capable of suppressing feeding behaviour, then bees that consumed a dose of 0.878-3.57 μg/bee/day would also exhibit depressed feeding; however, this was not the case. In addition, honeybees decreased the total volume of food that they consumed over the pre-exposure period when pre-exposed to nicotine for 3 days in both 0.5 M and 1.5 M sucrose, whereas this was not observed in honeybees pre-exposed to nicotine for 3 days in 1.0 M sucrose, indicating that the concentration of sucrose that nicotine is administered in does not account for the reduced feeding behaviour observed in the 0.5 M and 1.5 M sucrose treatments. i.e. if the bitter taste of nicotine was causing the bees to consume less solution, then this would also be apparent in honeybees pre-exposed to nicotine in 1.0 M sucrose; however, again, this was not the case.

Although intermittent schedules of nicotine administration may also be a factor in whether nicotine functions to alter the total volume consumed by honeybees over the pre-exposure period, given that variability is observed when the schedules of administration are consistent (i.e. the total volume consumed varies between treatments when honeybees are pre-exposed to nicotine chronically for 3 days) it is not possible to accurately assess how intermittent schedules of nicotine administration may impact the feeding behaviour of

honeybees in comparison to chronic nicotine pre-exposure at this time. Indeed, at present, it is not clear why some pre-exposure treatments in this study saw honeybees reduce the total volume of food that they consume, whilst others did not. Furthermore, honeybees that had been pre-exposed to 25 μM nicotine in 1.0 M sucrose for three days were instead shown to increase the total volume of solution consumed over the choice day in comparison to honeybees that were pre-exposed to sucrose alone. However, again, given that the average dose consumed across the pre-exposure period and the choice period was approximately equal to that consumed in other pre-exposure schedules (e.g. Pre-exposed to 25 μM nicotine in 1.0 M sucrose: Pre-exposed dose = 0.878 μg/bee/day, chosen dose = 0.566 ug/be/day. Pre-exposed to 25 μM and 100 μM nicotine in 1.5 M sucrose: Pre-exposed dose = 0.448-2.00 ug/bee/day. Chosen dose = 0.423-1.47 ug/bee/day), it is again unclear why honeybees chose to consume a greater total volume of solution over the choice period in this instance.

In contrast to honeybees that were pre-exposed to nicotine, unexposed honeybees that consumed nicotine over a 24 h two-way choice period were consistently shown to consume an equivalent volume of food to their sucrose-only controls. This was consistent regardless of the sucrose molarity that nicotine was administered in, the concentration of nicotine they were provided, and the dose of nicotine that the bees consumed during the 24 h choice period. In addition, it was clear that when honeybees found nicotine aversive (i.e. 250 μM and 500 μM nicotine in 0.5 M sucrose), they would preferentially consume from the tube that contained sucrose alone in order to meet their daily carbohydrate needs. This is in agreement with previous studies, where honeybees that are offered a 24 h two-choice test between toxic secondary metabolites in sucrose, or sucrose alone, will reject high concentrations of toxic substances and preferentially consume from the sucrose-only solution to meet their daily carbohydrate needs (Desmedt et al. 2016). Thus, honeybees that are offered a 24 h two-way choice between nicotine in sucrose, or equimolar sucrose, do not consume more or less solution than bees that feed on sucrose alone, indicating that nicotine did not promote or suppress feeding in any of these treatment groups. Therefore, if nicotine functions to suppress feeding in the honeybee, this effect is only observed when honeybees are forced to chronically or intermittently consume nicotine in their only source of food over a number of days.

To the best of my knowledge, only two studies exist that have assessed whether nicotine affects the total volume of food consumed by the honeybee. Interestingly, these studies also obtained variable results. Köhler et al. (2012a) noted that caged honeybees decreased the total volume of solution they consumed over a 7 day period when they were fed a diet of 300 μM nicotine in a 0.63 M sucrose solution, in comparison to honeybees that were fed sucrose alone. In contrast, a follow-on study by the same laboratory, using identical concentrations of nicotine and sucrose and an identical methodology to that used in their earlier study, observed that honeybees did not decrease the total volume they consumed over a 7 day period, in comparison to honeybees that were provided with sucrose alone (Köhler et al. 2012b). In other words, when the same experimental paradigm was repeated, they obtained conflicting results. Interestingly, studies that have assessed whether nicotine functions to suppress feeding in mammals have also obtained variable results. For instance, over a 2-20 day period of nicotine administration in mammals, nicotine has been shown to reduce food consumption (Wellman et al. 2005; Seoane-Collazo et al. 2014), increase food consumption (Perkins, 1992; Faraday et al. 2001), decrease food consumption on some days but not on others (Bellinger et al. 2010) or have no effect on the amount of food consumed (Rupprecht et al. 2016; Rupprecht et al. 2018). In other studies, administration of nicotine has been shown to decrease the amount of food rodents consume initially; however, the rodents will return to baseline levels of feeding over a matter of days, suggesting that if nicotine does function to suppress feeding behaviour in mammals, tolerance may develop to nicotine's feeding suppressant effects very rapidly (Caggiula et al. 1991; Bunney et al. 2016). This latter point is particularly interesting given that honeybees pre-exposed to nicotine typically displayed only a transient decrease in the total volume they consumed before their total consumption levels quickly returned to equivalent levels of their sucrose-only control groups. Although this may indicate that honeybees can develop tolerance to the suppressant effect that nicotine has on their feeding behaviour in some instances, given the variability of the results obtained across pre-exposure treatments (i.e. nicotine did not consistently suppress honeybee feeding behaviour), this cannot be said with any certainty at this time.

At present, it is not clear how, or indeed if nicotine functions as a feeding suppressant in mammals and substantial evidence exists both for and against the role of nicotine as a direct feeding suppressant. For instance, despite the fact that human smokers

tend to weigh less (Perkins et al. 1991; Perkins, 1992), clinical studies have shown that smokers and non-smokers have an equal caloric intake (Perkins et al. 1991; Perkins, 1992). Furthermore, rodent studies have identified that nicotine can suppress weight gain in rodents, independent of calorie intake (Rupprecht et al. 2016; Calarco et al. 2017; Rupprecht et al. 2018). Given that nicotine is known to increase the basal metabolic rate of mammals (McGovern and Benowitz, 2011), one explanation for these results is that nicotine doesn't function as a direct appetite suppressant *per se*, but instead suppresses weight gain by raising the basal metabolic rate of the animal whilst blunting the expected calorific increase that would typically arise from an increase in metabolic rate (McGovern and Benowitz, 2011). Despite this, evidence also exists which instead suggests that nicotine can function directly to suppress feeding in mammals by binding to nicotinic acetylcholine receptors (nAChRs) expressed in neurons mediating satiation, resulting in a direct suppression of the animals' appetite, and therefore a reduction in the amount of food consumed (Mineur et al. 2012). In addition, high doses of nicotine can also function in an indirect manner to reduce appetite by generating a state of malaise in mammals (Mishra et al. 2015). This suggests that nicotine may also function in a similar manner in the honeybee, altering their metabolic rate, or acting either directly or indirectly to mediate satiation or generate a state of malaise, which may account for the suppressed feeding observed in some instances in this study.

Honeybees that have been chronically fed 300 μM nicotine dissolved in a 0.63 M sucrose solution have been shown to have an increased basal metabolic rate to bees that are fed sucrose alone due to the energetic investment required in detoxifying nicotine (Du Rand et al. 2015). This suggests that honeybees that consume nicotine should increase the total amount of food they consume to meet this increased energetic demand; however, honeybees that consumed nicotine in the unexposed treatment groups did not increase their total consumption levels relative to their sucrose-only controls. This indicates that, at least in a 24 h two-way choice setting, consumption of nicotine at doses as high as 13.5 μg/bee/day does not result in increased carbohydrate consumption in the honeybee. Furthermore, honeybees that were pre-exposed to nicotine for a number of days either decreased their total consumption of food in comparison to sucrose-only control bees, or their total consumption levels remained unchanged to honeybees that consumed sucrose alone. Indeed, this is perhaps unsurprising, given that nicotine was present in the honeybees

only food source during the pre-exposure periods, and therefore increasing their total consumption in this situation would be counterproductive as this would simultaneously increase the nicotine dose the bees consumed. Despite this, honeybees that were shown to reduce their feeding during the pre-exposure period (i.e. were likely in a semi-starved state to that of the sucrose-only controls) did not subsequently increase the total volume of food that they consumed when they were provided with the opportunity to feed on sucrose alone during the 24 h two-way choice period to make up for this deficit, indicating that nicotine was not promoting carbohydrate consumption in these instances. Therefore, it appears that similar to mammals, nicotine increases the basal metabolic rate of honeybees; however, there is no concomitant increase in calorific intake to adjust to the metabolic demand of detoxifying the compound.

At present, the neural pathways involved in mediating satiation in insects are only beginning to be understood (Lin et al. 2019); however, certain pathways involved in mediating satiation have been found to be under cholinergic control (Yapici et al. 2016), indicating a possible means for nicotine to modulate feeding behaviour directly in insects through interaction with nAChRs. In addition, previous studies have identified that nicotine can generate a state of malaise in honeybees at concentrations as low as 10 nM (a dose of just 0.252 ng/bee/day) (Williamson et al. 2014), and malaise is known to suppress feeding behaviour in these insects (Ayestaran et al. 2010; Wright et al. 2010). Therefore, if nicotine functions to suppress feeding in the honeybee, it appears most likely to be caused by interactions with neurons that mediate appetite or due to a state of malaise (however, I hypothesise that the former is more likely, as honeybees may become tolerant to the aversive effects of nicotine, discussed below). Indeed, it is not possible at this time to conclude why some honeybees that were pre-exposed to nicotine decreased the total volume of food that they consumed over the pre-exposure period, and further studies are required to understand how nicotine administration modulates honeybee feeding behaviour, e.g. by assessing if honeybees are experiencing a state of malaise during the pre-exposure periods due to chronically consuming nicotine in their only source of food, or by assessing nicotine's ability to interfere with pathways mediating satiation.

Despite the fact that nicotine appears to depress feeding in honeybees in some circumstances, this does not necessarily impact the honeybees ability to function as a model

for nicotine addiction. Indeed, honeybees that consumed nicotine following the I48 schedule of administration were shown to reduce their feeding relative to the sucrose-only control over the choice day, despite this, these bees displayed a significant preference for 100 μM nicotine over this time, indicating that preferences for nicotine can arise even when the chemical is suppressing the overall feeding behaviour of the bee.

3.4.3 Unexposed honeybees do not display a preference for nicotine

Only a single study has examined whether adult worker honeybees exhibit a preference for nicotine in food. Singaravelan et al. (2005) identified that free-flying honeybees preferentially consume low concentrations of nicotine (3-15 μM) delivered in a 0.6 M sucrose solution during a 1 h two-way choice period. These bees were not pre-exposed to nicotine prior to the test. These data indicate that nicotine functions as a positive reinforcer in this insect. This same study identified that nicotine can also be aversive at high concentrations (30-120 μM) (Singaravelan et al. 2005).

In contrast to the study of Singaravelan et al. (2005), the bees in this experiment did not exhibit aversion to food containing nicotine in a 24 h two-way choice unless they were given concentrations ≥ 250 μM. Indeed, no aversion was seen for low concentrations of nicotine (e.g. 25 μM or 100 μM) in any of the experiments conducted, regardless of the sucrose background used. This suggests that honeybees aversion for nicotine differs based on the length of access to the drug.

One possible explanation for the difference in the concentrations that produced aversion in my study compared to Singaravelan et al. (2005) is the development of acute tolerance to the negative effects of nicotine. Acute tolerance can be defined as a decreased response to the effects of nicotine following previous acute exposure that typically develops over ~30 min to 2 h (Stolerman et al. 1973; Stolerman et al. 1974; Russel et al. 1990; Rosecrans et al. 1995; Prus et al. 2007; Govind et al. 2009; Dani et al. 2000). For instance, nicotine naïve rats exposed to a single high dose injection of nicotine tend to display depressed locomotion, believed to represent the adverse effects of high doses of the drug in these animals (Stolerman et al. 1973; Stolerman et al. 1974). However, if rats have received a separate injection of nicotine 2 h, but not 1 h prior, then the depressant effects of the second nicotine injection are blocked (Stolerman et al. 1973; Stolerman et al.

1974). In other words, once an animal has experienced nicotine, acute tolerance gradually develops over a number of hours, and a second encounter with nicotine has a diminished negative effect. This phenomenon is likely to be a result of nAChR desensitisation (Robinson et al. 2007). I predict that the free-flying honeybees in Singaravelen et al. (2005) found > 30 μM nicotine aversive because they were in a choice setting that lasted only one hour. In my experiments, unexposed honeybees allowed 24 h access to the drug may generate acute tolerance to the adverse effects of the drug resulting in a measured overall indifference. Indeed, evidence exists to indicate that nicotine functions to desensitise honeybee nAChRs, similar to what occurs in mammals and in a similar time course (Goldberg et al. 1999; Barbara et al. 2008; Dupuis et al. 2011).

Aversion to nicotine in both humans and rodent models is typically due to malaise incurred by high doses of the drug (Duke et al. 2015, Anderson and Bruznell, 2015) or due to the bitter taste of the drug when administered in oral studies (Hummel et al. 1992; Meliska et al. 1995; Nesil et al. 2011). These mechanisms may also be at work in the honeybee. Previous research has shown that 1 mM nicotine activates gustatory receptor neurons in the honeybee's mouthparts (Kessler et al. 2015). In addition, acute ingestion of concentrations as low as 10 nM nicotine, a dose of just 0.252 ng/bee/day, decreases the likelihood of honeybees displaying the righting reflex (Williamson et al. 2014) and increases the rate of grooming behaviour (Williamson et al. 2014). Both of these traits are associated with malaise in the bee (Hurst et al. 2014). As such, nicotine aversion in the honeybee is likely mediated either pre-ingestively, possibly by functioning as a bitter tastant, and/or post-ingestively resulting in a malaise-like state. Indeed, nicotine is known to result in a concentration-dependent aversion in both *Drosophila* (~4 mM in 35 mM fructose) (Sellier et al. 2011) and the bumblebee (100 μM in 0.5 M sucrose) (Teideken et al. 2014). Therefore, honeybees may have avoided concentrations of nicotine ≥ 250 μM due to malaise brought on by consuming the alkaloid and/or due to the bitter taste of the compound.

It is important to note that nicotine, unlike other drugs of abuse, is generally considered both hedonic and noxious to drug naïve animals (Fowler and Kenny, 2011). For instance, in monkeys, the dose of the drug that supports maximal rates of responding also induces vomiting when the drug-taking habit is being acquired (Goldberg and Spealman,

1982). Similar reports of nausea are also reported in response to initial cigarette use in humans (Zabor et al. 2013). Indeed, nicotine's immediate effects in drug naïve animals are clearly dose-dependent: too-little nicotine is insufficient to activate reward circuitry, whereas too-much nicotine results in seizures and, as such, is aversive (Cohen and George, 2013; Anderson and Bruznell, 2015; George and Koob, 2017; Wolfman et al. 2018). It is only a narrow range of nicotine doses that function as a positive reinforcer in animals (George and Koob, 2017), and even within this range, negative effects may be present (Fowler and Kenny, 2011). As such it would not be surprising if honeybees displayed an initial aversion to specific concentrations of the drug that subsequently develop into either indifference, or indeed preferences, following extended experience with the drug.

The unexposed honeybees' apparent indifference to 25 μM and 100 μM nicotine over the 24 h choice period is in agreement with both human and mammalian studies of nicotine addiction. It is well known that smokers do not immediately acquire a heavy smoking habit following administration of a small number of cigarettes (Dar and Frenk, 2010). Even so-called "chippers"; social smokers who sporadically smoke, sometimes as many as 20 cigarettes a week, are not motivated to administer a high number of cigarettes over a 24 h period (Shiffman, 1989; Brauer et al. 1996; Coggins et al. 2009). Instead, the motivation to self-administer a high number of daily cigarettes is only attained following the gradual development of nicotine dependence, brought about by a prolonged (weeks to months) exposure to nicotine (Shadel et al. 2000; DiFranza et al. 2000; Dar and Frenk, 2010; Schane et al. 2010). In agreement, mammalian studies have identified that rodents exhibit only a passive interest to press a lever that administers intravenous injections of nicotine upon the first presentation of the drug, whereas robust responding for nicotine only becomes apparent when rodents have been allowed extended daily access to nicotine (e.g. 23 h access a day for ~7-14 days) (Valentine et al. 1997; Fu et al. 2001; Brower et al. 2002; Fu et al. 2003; Parker et al. 2004).

Similar to mammals, unexposed honeybees were shown to be indifferent to both 25 μM and 100 μM nicotine over 24 h choice period. Although honeybees voluntarily consumed nicotine over this period, they did not consume sufficient volumes of the drug to exhibit an overall preference over sucrose alone. As such, it appears that although nicotine functions as a positive reinforcer in free-flying honeybees over short periods of

time (Singaravelan et al. 2005), caged honeybees do not form a measurable preference for nicotine within 24 h. In order to establish the ontogeny of nicotine preference over time, future experiments may look to examine nicotine preference over a narrower range of time points. Indeed, a caged capacitance-based feeding system has recently been developed in the Wright laboratory (Unpublished data), which allows for high-resolution (in the order of ms) quantification of the bees feeding behaviour throughout a 24 h period and is expected to be used in future studies.

3.4.4 Changing the concentration of sucrose the drug is administered in does not affect the preference for nicotine in the honeybee

Previous studies have indicated that bees are less likely to reject alkaloids when presented in more concentrated sucrose solutions (Gegear et al. 2007; Köhler et al. 2012a), presumably, as an increased concentration of sucrose is more effective at masking the bitter taste of these compounds (Cocco and Glendinning, 2012; Köhler et al. 2012a). For instance, Köhler et al. (2012a) identified that free-flying honeybees presented with a 10-way choice between a range of nicotine concentrations delivered in 0.15 M sucrose, found 30 μM nicotine aversive. In comparison, bees presented with a 10-way choice to the same nicotine concentrations presented in 0.6 M sucrose only displayed an aversion to nicotine at concentrations $\geq$ 150 μM. (Köhler et al. 2012a). Indeed, oral studies of nicotine in rodent models have similarly indicated that masking the taste of nicotine in water through the addition of sucrose or saccharin can increase the total volume of nicotine voluntarily consumed by the rodent (Smith and Roberts, 1995; Robinson et al. 1996; Kasten et al. 2016).

In order to assess whether sucrose molarity affected the preference for nicotine in caged honeybees during the 3 day pre-exposure period, experiments were repeated in a range of sucrose concentrations. However, increasing the sucrose concentration did not result in preferential consumption of nicotine over sucrose in any of the experiments conducted, regardless of the exposure protocol used (Appendix: Supplementary Fig. 1.1).

Indeed, a primary confounding variable in this study is that total consumption, and therefore dose decreases as the sucrose concentration increases. This particular factor has

not been considered in previous studies (Köhler et al. 2012a). As discussed above, insects regulate their intake of macronutrients to reflect specific optima (Simpson and Raubenheimer, 2012). For this reason, the dose of nicotine consumed by the honeybees depended in part on the total amount of carbohydrate the cohort of honeybees needed to stay alive each day. Future studies may therefore assess whether taste palatability impacts preferential consumption of nicotine by instead augmenting the taste qualities of the solution through a sweet but non-nutritious sweetener such as saccharin (Burke and Waddell, 2011). This would allow the taste of nicotine to be masked without affecting the honeybee's nutritional requirements, and therefore the dose of nicotine consumed by the bee.

3.4.5 Honeybees do not display a preference for nicotine following constant pre-exposure but display a significant preference following the I48 schedule

Rodent studies have identified that preferences for nicotine can be expedited by introducing a period of forced pre-exposure (~7 days) (Shoaib et al. 1997; Adriani et al. 2003; Natividad et al. 2013; Neugebauer et al. 2014; Renda and Nashmi, 2014). This 'primes' the rodent into an addicted state by facilitating the neurological changes responsible for nicotine addiction (e.g. nAChR upregulation) in advance of the self-administration session (Nguyen et al. 2004; Gould et al. 2014; Renda and Nasmi, 2014).

In order to assess whether honeybees could similarly be 'primed' into an addicted state, honeybees were pre-exposed to nicotine for a period of 3 or 5 days before being presented with a two-way choice for the drug. The 3 day pre-exposure period was predicted to be sufficient in facilitating the neurological changes responsible for nicotine dependence, as a recent study has indicated that nAChRs are upregulated in the bee in response to chronic oral consumption of 30-300 μM nicotine after 48 h (Christen et al. 2016). The 5 day pre-exposure period was included as extended periods of nicotine administration in rodents are known to increase the level of dependency, as evidenced by increased symptoms and duration of withdrawal syndrome (Damaj et al. 2003; Skjei and Markou, 2003; Vann et al. 2006).

Honeybees did not display a preference for 25 μM or 100 μM nicotine regardless of the pre-exposure length and regardless of the sucrose molarity the drug was administered in. Although at first glance this suggests that neither 3 or 5 days pre-exposure is sufficient to result in nicotine dependence in the bee, these results are perhaps best viewed in light of the results of the I48 schedule, which resulted in a significant preference for 25 μM nicotine.

The I48 schedule is essentially identical to the 3 day chronic administration protocol, with the exception of a single forced period of nicotine abstinence. That is, the initial 48 h of both treatments are identical, and the protocols differ in respect of whether bees received a further 24 h chronic nicotine period (3 day pre-exposure), or instead a 24 h period of forced abstinence (I48, 24 h of sucrose alone). The fact that honeybees preferred 25 μM nicotine in the I48 schedule indicates that the neurological changes that occur within the initial 48 h of nicotine exposure are sufficient in the development of a preference for nicotine following a period of abstinence.

In rodent models, forcing nicotine-dependent animals to undergo a 24 h period of nicotine abstinence (i.e. withdrawal) is known to result in a transient but robust increase in nicotine self-administration following the reintroduction of the drug (O'Dell and Koob, 2007; George et al. 2007; Nesil et al. 2011; Cohen et al. 2012; Gilpin et al. 2014; Cohen et al. 2015) a response termed the "nicotine deprivation effect" (NDE) (See: General introduction). This effect is known to be very specifically under the control of corticotropin releasing factor (CRF) (George et al. 2007; Cohen et al. 2012; Grieder et al. 2014; Cohen et al. 2015; Zhao-Shea et al. 2015), as blockade of CRF during the 24 h abstinence period abolishes both withdrawal symptoms in response to nicotine cessation, and subsequently the NDE upon drug reintroduction (Geroge et al. 2007; Bruijnzeel et al. 2007; Marcinkiewcz et al. 2009; Bruijnzeel et al. 2009; Grieder et al. 2014; Cohen et al. 2015; Zhao-Shea et al. 2015).

Withdrawal from nicotine is known to result in decreased dopamine release in reward pathways of the rodent brain (Rada et al. 2001; Natividad et al. 2010; Zhang et al. 2012; Grieder et al. 2012; Grieder et al. 2014; Zhao-Shea et al. 2015). This effect is believed to be synonymous with anhedonia, stress, and depression (Belujon and Grace,

2017). These dopamine deficits have recently been shown to be directly mediated by CRF expression within dopaminergic neurons themselves (Grieder et al. 2014; Zhao-Shea et al. 2015). Indeed, specific knock-down of CRF expression within dopaminergic neurons can completely abolish nicotine withdrawal in the rodent (Grieder et al. 2014; Zhao-Shea et al. 2015), indicating the importance of dopaminergic-CRF upregulation in maintaining nicotine addiction. As such, 24 h periods of nicotine cessation in rodents results in CRF-mediated dopamine deficits. Thus, the 'drug-loading' behaviour observed following periods of nicotine deprivation is mediated by a desire to return dopaminergic function to normality (George and Koob, 2017). In an addicted animal, this is an effect that is readily achieved by nicotine administration (Zhang et al. 2012). As such, the animal learns that in order to avoid withdrawal syndrome, they must self-administer nicotine, i.e. negative reinforcement. Furthermore, it appears that simply returning to pre-abstinent nicotine levels of self-administration is not sufficient, and instead, animals must 'drug-load' in order to overcome withdrawal syndrome. Given that both the 3 day pre-exposed and I48 treated bees both experience a minimum of 48 h chronic pre-exposure to nicotine, suggests that the neurological changes that occur during the 48 h exposure period are sufficient to generate a dependent state in the bee. The fact that honeybees exhibit a significant preference for nicotine only following a period of 24 h nicotine abstinence (I48) and not following continued chronic exposure (3 day pre-exposure) suggests that negative reinforcement and 'drug-loading' behaviour is required in order to identify a preference for nicotine during a 24 h two-way choice period. This suggests then that the indifference to nicotine observed in the 3 day pre-exposed bees reflects the bees ideal chosen dose, i.e. the dose that maintains the desired level of haemolymph nicotine to avoid 'withdrawal' whilst remaining below the level of adverse or toxic effects. Although not specifically tested in this study, if honeybees are simply maintaining their desired dose, then reducing the nicotine concentration during the 24 h choice period (e.g. from 25 μM to 5 μM), should result in preferential consumption from the nicotine-containing tube in order to compensate for the reduction in nicotine concentration, an effect that has been observed in mammalian models (Adriani et al. 2002a; Adriani et al. 2002b; Harris et al. 2011).

Addiction studies in invertebrates are still in their infancy, and the function of withdrawal has been largely overlooked in invertebrate models (Landayan and Wolf, 2015; Lowenstein and Velazquez-Ulloa, 2018; Ryvkin et al. 2018). Indeed, although the function

of CRF in mediating stress responses in invertebrates appears conserved (Lee et al. 2008; Liu et al. 2011; Jee et al. 2013; Mohammad et al. 2016; Mohorianu et al. 2017; Ketchesin et al. 2017; Herb et al. 2018), only a single paper has studied the effects of CRF and withdrawal in an invertebrate model. Jee et al. (2013) identified that nematodes that undergo forced abstinence from ethanol display robust withdrawal symptoms as evidenced by increased tremors. However, this effect could be blocked in animals that lacked CRF expression (Jee et al. 2013), a result in line with mammalian models of ethanol withdrawal (de Guglielmo et al. 2017). In addition, recently, it has been identified that insects express the CRF ortholog within their dopaminergic neurons (Davie et al. 2017; Croset et al. 2018). Indeed, dopamine deficits in insects result in a remarkably similar 'anhedonic' state to that observed in mammals (Riemensperger et al. 2011). Collectively this suggests that the molecular 'blue-print' for nicotine withdrawal syndrome is present in the insect. However, further studies are required to assess whether honeybees exhibit withdrawal symptoms during the nicotine abstinence period and if the apparent drug-loading behaviour observed is also under the control of CRF-mediated dopamine deficits, as it is in the mammal.

3.4.6 Honeybees display a preference for nicotine following 12 h intermittent schedules of administration

The I12 schedule was included to more accurately reflect the human condition. Smokers are known to undergo a period of nicotine abstinence during overnight sleep (Matta et al. 2006), which results in a partial decline in nicotine plasma levels (Matta et al. 2006), allowing recovery of nAChR desensitisation (Benowitz, 2009; Benowitz, 2010; Dani et al. 2015), and ultimately resulting in withdrawal and negative reinforcement over the early stages of the morning (Baker et al. 2007). The importance of intermittent periods of abstinence in the development of nicotine dependence is reflected by the fact that intermittent periods of abstinence can result in profoundly different neurological changes to that observed in chronic administration paradigms (Moretti et al. 2010; Allain et al. 2015; George and Koob, 2017). For this reason, continuous access paradigms do not correctly reflect the intermittent nature of nicotine administration in human subjects (Cohen and George, 2013).

Unlike humans, honeybees do not experience prolonged periods of sleep and instead take bouts of sleep lasting ~40 s (Klein et al. 2008). Indeed, only ~50% of foragers are asleep overnight at any one time (Klein et al. 2014). As such, it was predicted that honeybees would continue to consume nicotine overnight, and therefore the chronic paradigms used would not accurately reflect the human condition.

The intermittent data predicts that honeybees in the 3 day or 5 day pre-exposure period did not experience prolonged periods of nicotine abstinence. For example, in the I12 schedule, bees consumed ~ 35 µl during the day (9 am – 9 pm coinciding with nicotine administration). At night (sucrose administration from 9 pm – 9 am), this amount was reduced by only 10 µl to ~25 µl. The result of scheduling an overnight period without nicotine produced a 'withdrawal' period, causing bees to exhibit a preference for the 100 µM solution during the subsequent two-choice test.

Only a handful of papers have examined 'human equivalent' paradigms in rodents at present. Exposure to nicotine vapour for 12 h intermittent periods has been shown to increase rodents' self-administration of nicotine (i.e. the NDE) (Gilpin et al. 2014). Furthermore, nicotine self-administration was shown to increase as a function of the intermittent exposure cycle (Gilpin et al. 2014). i.e. the more 12 h intermittent periods the rodent experienced, the greater the propensity to self-administer the drug. Indeed, 14 h intermittent periods in rodents lead to CRF mediated withdrawal symptoms that are more robust in comparison to continual nicotine delivery (George et al. 2010; Baiamonte et al. 2014; Brynildsen et al. 2016). This is in line with the suggestion that repeated intermittent access schedules 'sensitise' stress response pathways, resulting in increased negative reinforcement behaviour (George and Koob, 2017). Given that honeybees pre-exposed to 100 µM nicotine in the I12 schedule of administration showed a significant preference for the drug, suggests that similar to mammals, overnight periods of abstinence may result in a 'sensitised' (i.e. hyperresponsive) stress response following periods of nicotine abstinence, ultimately resulting in negative reinforcement behaviour which is observable as an overall preference for the nicotine-containing food during the 24 h two-way choice period.

A critical caveat to both the I12 and I48 schedules of nicotine administration is that symptoms of withdrawal were not directly assessed and are simply inferred from the honeybees' preferential nicotine consumption behaviour. Withdrawal-like symptoms in the honeybee have been shown in response to abstinence from both cocaine (Barron et al. 2009) and morphine (Fu et al. 2013); however, they have not yet been assessed in response to nicotine. Importantly, withdrawal-like symptoms resulting from nicotine cessation have been observed in worms in a number of studies (Feng et al. 2006; Rawls et al. 2011; Polli et al. 2015; Bach et al. 2016), suggesting that nicotine is capable of generating withdrawal in invertebrates. Future studies will need to confirm that withdrawal-like behaviour coincides with periods of nicotine abstinence in the honeybee and that withdrawal symptomology is responsible for the preferential consumption of nicotine following periods of drug abstinence.

3.4.7 Preferences for nicotine in the intermittent schedules occur in a schedule and concentration-dependent manner

The preferences for nicotine in both the I12 and I48 intermittent schedules were concentration-dependent. Thus, bees fed 100 μM nicotine solutions in the I12 group exhibited the strongest preference for nicotine during the choice period. In contrast, bees fed 25 μM nicotine solutions in the I48 group exhibited the strongest preference in the choice period. Of note, however, is the general trend observed for an increase in preference over both nicotine concentrations in bees pre-exposed to the I12 schedule. It is not currently clear at present what could account for the differences between these two schedules of administration. Indeed, the importance of schedules of administration in addiction research in mammals has only become apparent in the last decade, and the consequences of these schedules on the underlying neurocircuitry are at present complex and unclear (Moretti et al. 2010; Allain et al. 2015). However, given that rodents exhibit a greater propensity to self-administer nicotine as a function of intermittent exposure cycle frequency (discussed above), the trend observed in honeybees in the I12 schedule may be due to the increased number of intermittent cycles experienced by these bees. Future studies may look to increase the number of periods of nicotine abstinence in the I48 schedule to better understand the relationship between frequency of intermittent access and the concentration of nicotine preferred.

Recent studies in the honeybee have indicated that haemolymph nicotine has a relatively low clearance rate (~25 % after 24 h following 3 days of chronic 300 μM nicotine consumption) (Du Rand et al. 2017). As such, nicotine levels are likely to decline at different rates in the I48 and I12 schedules, and the rate of this will be dependent on both the initial dose consumed and the duration of abstinence (Du Rand et al. 2017). Further studies focused on identifying the underlying molecular and neurological changes that occur over these different schedules (e.g. nAChR and CRF expression) would be beneficial in elucidating the cause of differential nicotine preferences following intermittent schedules of nicotine exposure.

3.4.8 Pre-exposure to nicotine affects the nicotine dose chosen in the test period

In all cases, during the 24 h two-way choice period, honeybees selected a lower dose of nicotine than they had experienced over the 3 or 5 day pre-exposure periods, indicating that honeybees were not attempting to match the dose they were previously accustomed to. This is in agreement with oral studies in rodents which have identified that although rodents increase their total dose when undergoing forced pre-exposure, they subsequently decrease their dose when presented with a two-way choice for the drug (Todte et al. 2001; Pawlak and. Schwarting, 2002; Dadmarz and Vogel, 2003). This suggests that animals will consume high doses of nicotine when forced too to obtain their water requirements but find prolonged forced consumption of such high doses aversive (Isiegas et al. 2009; Lee et al. 2017).

3.4.9 Nicotine does not affect mortality

Nicotine administration did not result in increased mortality relative to control in any of the experiments conducted. This result is in agreement with previous studies, which have indicated that concentrations of nicotine as high as 300 μM administered chronically to honeybees in food for a period of 7 (Köhler et al. 2012b) to 15 days (Singaravelan et al. 2006) does not result in increased mortality. Indeed, the LD50 for nicotine in honeybees is extremely high: 12 mM after 48 h constant exposure to the drug in food (Detzel and Wink, 1993), and is similar to that observed in other insect models (*Drosophila*: LD50 of 20 mM after 36 h chronic exposure to the drug in food) (Matta et al. 2006). As such, the

concentrations of nicotine used in these experiments are well below the LD50 concentrations in the honeybee. The lack of mortality observed in response to nicotine further validates the use of feeding assays to study the addictive properties of nicotine in these insects.

3.4.10 Conclusion

To the best of my knowledge, this serves as the only study to date that has identified the importance of prolonged intermittent access procedures in attaining preferential drug consumption in an invertebrate model. The fact that intermittent procedures reveal a preference for nicotine indicates the importance of integrating negative reinforcement into future addiction studies in insects.

Unfortunately, the studies assessing whether increasing the sucrose concentration nicotine is administered in can augment preferential nicotine consumption in the honeybee were confounded by a simultaneous decline in the dose consumed. Therefore, it is not clear whether taste-masking effects will be fruitful to future research. Future studies may instead rely on the use of saccharin to assess if this is indeed the case.

Collectively, this study has indicated that honeybees display a significant preference for nicotine following intermittent pre-exposure to the drug. However, it is imperative to note that preferences for a compound do not indicate that an animal is addicted to the compound. In order to be characterised as an addict, sufficient evidence of maladaptive behaviour in line with the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) or the World Health Organizations International Classification of Diseases (ICD10) must be identified. Future studies may therefore focus on identifying whether honeybees exhibit symptoms of withdrawal during periods of nicotine abstinence, as hypothesised.

Chapter 4.0 Do bumblebees prefer to consume nicotine delivered in a sucrose solution over sucrose alone?

4.1 Introduction

Despite the overlapping ecological niche of bumblebees and honeybees as floral foragers, bumblebees and honeybees differ in a wide number of respects, such as brain size (Mares et al. 2005), genetics (Sadd et al. 2015), longevity (Röseler and van Honk, 1990; Johnson, 2010), number of xenobiotic detoxification enzymes (Sadd et al. 2015), their susceptibility to the toxic effect of pesticides (Piiroinen and Goulson, 2016; Manjon et al. 2018), and their preferential consumption of neonicotinoids (Kessler et al. 2015). This raises the question as to whether honeybees and bumblebees differ in their preferential consumption of nicotine. Indeed, different strains of rats are known to vary dramatically in their propensity to self-administer the drug (Shoaib et al. 1997; Cadoni, 2016). For instance, in comparison to Fischer rats, Lewis rats are more likely to find nicotine rewarding (Horan et al. 1997; Philibin et al. 2005) exhibit greater dopamine release in response to the drug (Sziraki et al. 2001) and exhibit more pronounced withdrawal symptoms following nicotine cessation (Suzuki et al. 1999). Given these marked differences, it is therefore desirable to identify if different species of bee may be more suitable for the study of addiction than others.

To date, only a handful of studies exist which have examined whether nicotine may function as a positive reinforcer in the bumblebee (Tiedeken et al. 2014; Barrachi et al. 2015; Palmer-Young et al. 2017; Barrachi et al. 2017a). These studies have largely focused on whether infected bumblebees preferentially consume nicotine as a means to self-medicate when undergoing an infection due to the antimicrobial nature of the drug (Pavia et al. 2000). For instance, Barrachi et al. (2015) identified that the buff-tailed bumblebee (*Bombus terrestris*) infected with the common gut parasite, *Crithidia bombi*, displayed a preference for low concentrations of nicotine in a caged environment when consumption was measured over the course of the insects' life. However, a preference for nicotine at equivalent concentrations was not observed in *B. terrestris* workers when it was measured over a 24 h period (Tiedeken et al. 2014) or in the closely related species, the common eastern bumblebee (*Bombus impatiens),* regardless of whether it had been infected with *C. bombi* (Palmer-Young et al. 2017). In a follow-on study, Barrachi et al. (2017a) identified

that uninfected free-flying buff-tailed bumblebees displayed a preference for artificial flowers containing low concentrations of nicotine dissolved in a sucrose solution. In addition, Barrachi et al. (2017a) identified that bumblebees avoid high concentrations of nicotine in a two-choice test, despite this, they were more likely to remain faithful to flowers that had previously contained nicotine, avoiding alternative flowers that contained sucrose, even when the nicotine solution was replaced with water. In addition to nicotine, preferences have been observed in the bumblebee over a 24 h period in response to the neonicotinoid pesticides imidacloprid and thiamethoxam (Kessler et al. 2015), which are chemically similar to nicotine. These studies, alongside the preferences for nicotine observed in chapter 3.0 in honeybees, suggest that nicotine may manipulate bee behaviour in a manner analogous to that observed in mammals (De Biasi and Dani, 2012), perhaps indicating that bumblebees could become addicted to nicotine.

The fidelity to sub-optimal flowers in bees that have experienced nicotine in the study of Barrachi et al. (2017a) indicates that nicotine interferes with the bees evaluation of valence, resulting in maladaptive choice behaviour. Behavioural economics combines basic concepts from economics and behavioural psychology to assess the relative motivation to pursue rewarding stimuli (Correia et al. 2010). In addiction studies, behavioural economics is applied to assess the motivation to pursue a drug reward over an alternative non-drug reinforcer (Correia et al. 2010). For example, in rodents, this is typically assessed by offering the rodent a choice between a drug or saccharin sweetened water (Ahmed et al. 2010, Ahmed, 2018). How reinforcing the drug is to a subject relative to the alternative reinforcer can then be determined by altering the magnitude of the alternative reinforcer whilst maintaining the level of drug reinforcement (Correia et al.2010; Ahmed et al. 2010, Ahmed, 2018). Thus, if nicotine interferes with the bumblebees evaluation of valence, then it would be expected that bumblebees that have previously experienced nicotine may make similar sub-optimal choices in a caged setting within the laboratory.

At present, the evidence that bumblebees choose to consume nicotine is contradictory and seems to depend on context. To date, none of these studies have tested what impact a period of forced pre-exposure has on the bumblebees preferential consumption of nicotine. Furthermore, although bumblebees were shown to remain faithful

to flowers associated with nicotine when they became the sub-optimal choice in a free-flight experiment, at present, no studies have examined whether bees will continue to consume nicotine when presented with an alternative reinforcer of greater nutritional value, indicative of maladaptive behaviour. In order to test whether bumblebees display a preference for nicotine, a subset of the pre-exposure tests undertaken with honeybees were repeated with bumblebees. Specifically, I tested whether forced pre-exposure affects an observed preference for nicotine in caged forager bumblebees. In addition, it was tested whether bumblebees display a preference for nicotine-laced solutions when they became the sub-optimal choice in terms of nutritional value.

4.2 Methods

Bumblebees were exposed to four different feeding schedules in these experiments: (i) Unexposed, (ii) pre-exposed for 3 days, (iii) pre-exposed for 5 days, or (iv) valence exposure (see general methods). The concentrations of sucrose and nicotine used in these experiments are detailed in table 4.2. Nicotine concentrations for the unexposed and 3 day pre-exposure assays were selected as they encompass the concentrations known to be both preferred and avoided in prior experiments in bumblebees (see table 1.5.3). Initial experiments assessing whether bumblebees displayed a preference for nicotine in 0.5 M sucrose used low nicotine concentrations (6.25 to 200 μM). However, as a marginal preference was observed for 100 μM nicotine in this study, later experiments, where nicotine was dissolved in 1.0 M sucrose, used a broader range of nicotine concentrations (25 to 1000 μM). As in chapter 3.0, sucrose concentrations for the unexposed and 3 day pre-exposure assays were selected as they encompass the total sugar concentrations found within *Nicotiana* spp. nectar (0.5 – 3.0 M) (Kessler et al. 2012; Tiedge and Lohaus, 2017; Tiedge and Lohaus, 2018). Similar to the earlier experiments in honeybees, the sucrose concentration that nicotine was administered in was varied to modulate the bitter taste of nicotine. Note that honeybee and bumblebee studies were conducted in parallel; therefore, it was not possible to use the results obtained in chapter 3.0 to guide experimental planning for the bumblebee nicotine studies conducted here.

Sucrose concentrations for the valence experiment were 1.1 M, 1.2 M, and 1.3 M. These concentrations were chosen as bees are known to prefer more concentrated sucrose solutions to less concentrated solutions (Konzmann and Lunau, 2014). Offering

bumblebees a more concentrated sucrose solution in comparison to nicotine dissolved in 1.0 M sucrose allowed assessment of the bumblebees motivation to consume nicotine in the face of an alternative reward of greater value.

Unexposed treatments were conducted once for each sucrose and nicotine concentration (table 4.2). In all cases, the unexposed experiments were run separately from the pre-exposed experiments, and the data obtained from the unexposed experiments was used to compare against all pre-exposure experiments. i.e. the data obtained for the 1.0 M unexposed treatment was used as a comparison to the 3 day pre-exposure and 5 day pre-exposure treatments. In addition, due to limited availability within incubators, the 5 day pre-exposure experiment was conducted separately to the 3 day pre-exposure experiment. To make this clear, the 3 day pre-exposure data is represented graphically with dashed lines. All treatments for the valence experiment were conducted at the same time, i.e. the 3 day pre-exposure treatment was repeated for this study.

Table 4.2 | Nicotine concentrations (µM) and sucrose concentrations (M) used in the bumblebee choice experiments.

Feeding Schedule	0.5 M sucrose	1.0 M sucrose
Unexposed	0, 6.25, 12.5, 25, 100, 200	0, 25, 100, 500, 1000
Pre-exposure: 3 day	0, 6.25, 12.5, 25, 100, 200	0, 25, 100, 500, 1000
Pre-exposure: 5 day	N/A	0, 100
Valence exposure	N/A	0, 100

4.3 Results

4.3.1 The response of unexposed and pre-exposed bumblebees to different concentrations of nicotine administered in 0.5 M sucrose

The amount of food consumed by bumblebees over the pre-exposure period depended on the time of measurement and nicotine concentration in the food (Fig. 4.3.1a. RM-GLM, Time x Concentration: $F_{(13.7, 474)} = 2.89$, $\eta_p^2 = 0.084$, $p = <0.001$). Although there is a general trend for increased total consumption in bees that are exposed to nicotine solutions, only the 100 μM nicotine treatment is significantly different from the 0 μM control over the first day of pre-exposure (Extended data table. 4.3.1.1a).

The data for the 24 h choice test show that exposure to nicotine in food for prolonged periods did not influence subsequent choice behaviour (Fig 4.3.1b) (GzLM, Concentration x treatment: $\chi^2_{(5)} = 3.27$ $p = 0.659$). Although there was a weak preference for the nicotine solution in bumblebees that had been pre-exposed to 100 μM, this did not reach significance (One sample t-test against 0 (Holm-Bonferroni), $p = 0.072$).

Bumblebees in the pre-exposed treatment group consumed a significantly lower total volume of solution during the 24 h choice period than the unexposed treatment group when they had been pre-exposed to no nicotine (0 μM), 6.25 μM, 12.5 μM, or 25 μM nicotine (Fig.4.3.1c) (Treatment x Concentration: $F_{(5, 280)}$, $= 2.76$, $\eta_p^2 = 0.047$, $p = 0.019$. Extended data table 4.3.1.1c). Interestingly, both pre-exposed bees and unexposed bees consumed similar total volumes of food when offered a choice between sucrose or 100 μM or 200 μM nicotine (Extended data table 4.3.1.1c). The amount consumed by bees pre-exposed to nicotine did not differ from the amount consumed by the 0 μM pre-exposed control group (Extended data table 4.3.1.1c), whereas unexposed bumblebees presented with a choice between sucrose or 100 or 200 μM nicotine consumed a lower total volume of solution than their sucrose-only (0 μM) control (*Post hoc* Bonferroni. 0 μM vs 100 p = 0.09, 0 μM vs 200 μM p = 0.058. Note marginal *p* values. Extended data table 4.3.1.1c)

Bumblebees: Three day pre-exposure to nicotine in 0.5 M sucrose

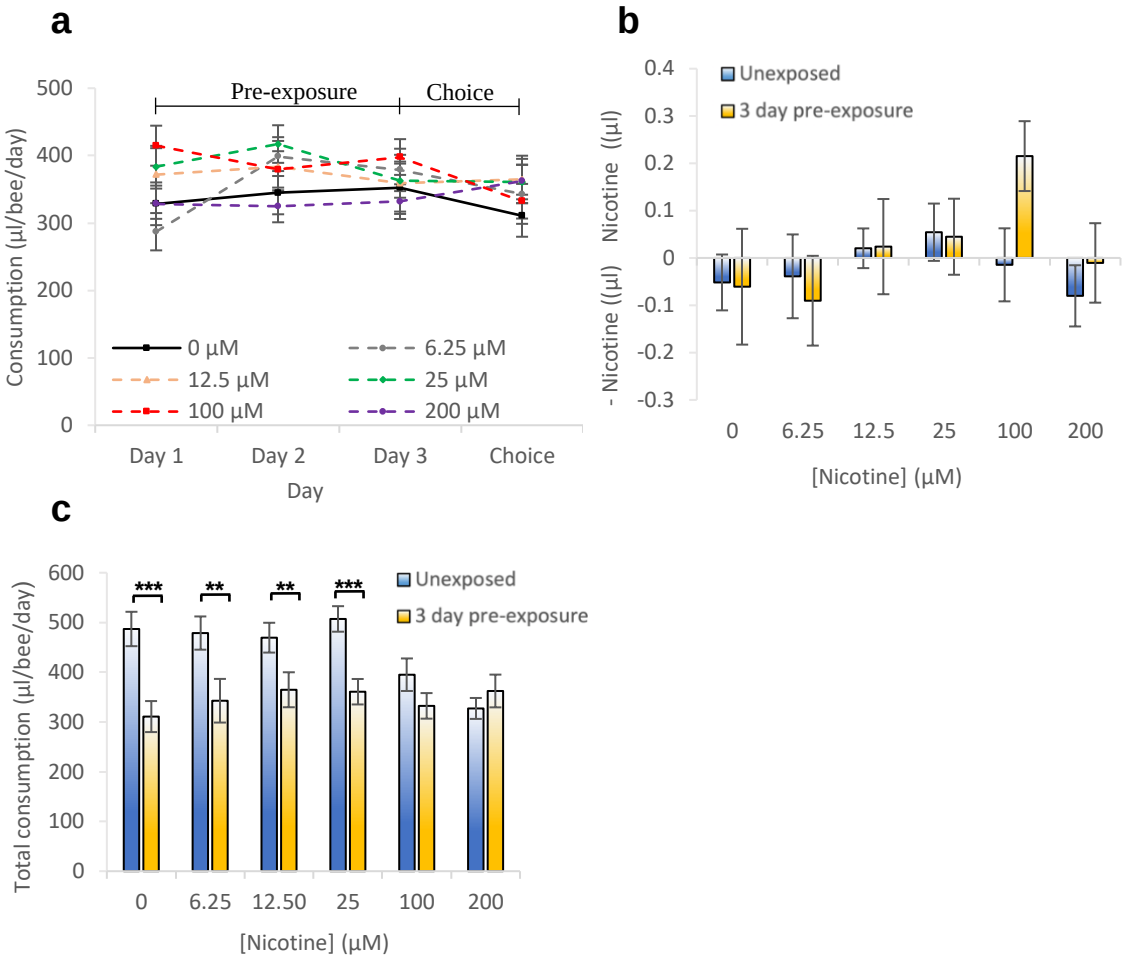

Figure 4.3.1 | Bumblebees did not display a preference for 100 µM nicotine following 3 days pre-exposure in 0.5 M sucrose. (a) Bumblebee pre-exposed to nicotine for a period of 3 days varied their total consumption as a function of nicotine concentration and time (Extended data table 4.3.1.1a). **(b)** Unexposed bumblebees did not display a preference for 100 µM, whereas a marginal preference for 100 µM nicotine is observed following 3 days pre-exposure (Extended data table 4.3.1.1b). Data represent the mean difference in the amount consumed over the 24 h period. Positive values indicate a preference for nicotine and negative avoidance of nicotine. **(c)** Total consumption across the 24 h two-way choice period varied depending on whether bumblebees were unexposed or pre-exposed to nicotine (Extended data table 4.3.1.1c); however, only unexposed bees presented with a choice between sucrose or 100 or 200 µM nicotine consumed a significantly lower total volume over the choice period from their sucrose-only control (0 µM) (Extended data table 4.3.1.1c. Note marginal *p* values). Asterisks indicate significant differences between treatments at each concentration. Bonferroni adjusted (***p* <0.01, *** *p* <0.001). Bars and line graphs indicate mean (±s.e.m.) of consumption (µl) per bee, controlled for by evaporation.

The total dose that the bees consumed over the course of the experiment was also measured (Dose table 4.3.1). The dose of nicotine among the pre-exposed bees was as much 2-30x greater than the lowest nicotine concentration, depending on the treatment (Welch's ANOVA, $F_{(4)} = 101$ $\eta_p^2 = 0.680$ $p = <0.001$. Dose table 4.3.1a). For example, bumblebees provided with 6.25 μM nicotine consumed a dose of 1.10 μg/bee/day, 95 % CI [0.949, 1.26], whereas those provided with 200 μM consumed an average dose of 32.7 μg/bee/day, 95 % CI [28.8, 36.6]. During the 24 h choice test, the pre-exposed bees consumed a significantly lower dose of nicotine than the unexposed bumblebees (GzLM, main effect of treatment, $\chi^2_{(1)} = 5.73$ $p = 0.017$. Extended data table 4.3.1.2d), with the exception of bumblebees that were pre-exposed to 200 μM nicotine, where they consumed a significantly higher dose of nicotine over the choice day than unexposed bees (Extended data table 4.3.1.2d). In all cases, the bees consumed less nicotine than they were exposed to during the 3 day pre-exposure period (Dose table 4.3.1e) (GzLM, Concentration x Time: $\chi^2_{(4)} = 1171$ $p = <0.001$). For example, bees pre-exposed to 100 μM nicotine consumed 20.0 μg/bee/day, 95 % CI [17.5, 22.6] during the pre-exposure period, but during the test, they chose to consume a dose of 9.48 μg/bee/day, 95 % CI [7.64, 11.3].

No unexposed bees or pre-exposed bees died during the course of the experiment.

Extended data table 4.3.1.1 | *B. terrestris* statistics for 3 day pre-exposure and 24 h two-way choice data in 0.5 M sucrose. Data correspond to figure 4.3.1a-c. **(a)** RM-GLM for the total consumed across the 3 day pre-exposure and 24 h two-way choice period. n: 0 μM (19), 6.25 μM (26), 12.5 μM (23), 25 μM (35), 100 μM (36) 200 μM (26). **(b)** GzLM for the indexed 24 h two-way choice day for unexposed and pre-exposed bumblebees, and one-sample t-tests against 0. n: Pre-exposed bees: 0 μM (23), 6.25 μM (30), 12.5 μM (26), 25 μM (37), 100 μM (39) 200 μM (30). Unexposed bees: 0 μM (18), 6.25 μM (18), 12.5 μM (18), 25 μM (18), 100 μM (17) 200 μM (18). **(c)** GLM for the total consumed during the 24 h two-way choice period for unexposed and pre-exposed bumblebees.

(a)†	Time: $F_{(2.75, 474)} = 2.98$, $\eta_p^2 = 0.019$, $p = 0.036$ Concentration: $F_{(5, 158)} = 0.677$, $\eta_p^2 = 0.021$, $p = 0.642$ Time x Concentration: $F_{(13.7, 474)} = 2.89$, $\eta_p^2 = 0.084$, $p = <0.001$ *Post hoc* (LSD) pairwise comparisons for time. Only significant effects are reported: Day 1 vs day 2 $p = 0.020$, day 2 vs day 4 $p = 0.014$. *Post hoc* (LSD) comparing nicotine treatments against control at each time point. Only significant effects are reported: Day 1: 0 μM vs 100 μM $p = 0.039$.
(b)	Concentration: $\chi^2_{(5)} = 5.78$ $p = 0.328$ Treatment: $\chi^2_{(1)} = 0.602$ $p = 0.438$ Concentration x treatment: $\chi^2_{(5)} = 3.27$ $p = 0.659$ One sample T-test against 0 (Holm-Bonferroni): Unexposed: 0 μM $T_{(17)} = -1.08$ $p = 1.000$, 6.25 μM $T_{(17)} = -4.39$ $p = 1.000$, 12.5 μM $T_{(17)} = 0.488$ $p = 1.000$, 25 μM $T_{(17)} = 0.897$ $p = 1.000$, 100 μM $T_{(16)} = -0.188$ $p = 1.000$, 200 μM $T_{(17)} = -1.24$ $p = 1.000$, 3 day 0 μM $T_{(22)} = -0.496$ $p = 1.000$, Pre-exposed: 6.25 μM $T_{(29)} = -0.953$ $p = 1.000$, 12.5 μM $T_{(25)} = 0.238$ $p = 1.000$, 25 μM $T_{(36)} = 0.558$ $p = 1.000$, 100 μM $T_{(38)} = 2.92$ $p = 0.072$, 200 μM $T_{(29)} = -0.125$ $p = 1.000$
(c)	Treatment: $F_{(1, 280)} = 31.6$, $\eta p2 = 0.102$, $p = <0.001$ Concentration: $F_{(5, 280)} = 2.08$, $\eta_p^2 = 0.036$, $p = 0.068$ Treatment x Concentration: $F_{(5, 280)} = 2.76$, $\eta_p^2 = 0.047$, $p = 0.019$ *Post hoc* pairwise comparisons between treatments at each concentration (Bonferroni): 0 μM $p = <0.001$, 6.25 μM $p = 0.005$, 12.5 μM $p = 0.008$, 25 μM $p = <0.001$, 100 μM $p = 0.077$, 200 μM $p = 0.438$ *Post hoc* pairwise comparisons between concentrations within each treatment (Bonferroni), only significant effects or marginal effects are reported: Unexposed: 0 μM vs 200 μM $p = 0.058$, 0 μM vs 100 μM $p = 0.09$, 25 μM vs 100 μM $p = 0.052$, 25 μM vs 200 μM $p = 0.008$.

† Mauchly's test indicated that the assumption of sphericity has been violated ($\chi^2_{(5)} = 39.5$, $p = <0.001$), therefore Huynh-Feldt corrected tests are reported ($\varepsilon = 0.915$).

Dose table 4.3.1 | Summary of the average dose consumed by *B. terrestris* following consumption of nicotine in the 24 h two-way choice periods and during the pre-exposure period. Data correspond to figure 4.3.1a-c. Values represent the average nicotine dose consumed per individual bumblebee (μg/bee/day) and their respective 95 % CIs.

[Nicotine] μM	Pre-exposure period dose		Chosen dose			
			Pre-exposed		Unexposed	
	μg/bee/day	95 % CI	μg/bee/day	95 % CI	μg/bee/day	95 % CI
6.25	1.10	0.949, 1.26	0.477	0.351, 0.602	0.680	0.559, 0.801
12.5	2.31	1.84, 2.78	1.01	0.754, 1.26	1.48	1.27, 1.68
25	4.83	4.25, 5.41	2.32	1.77, 2.86	3.37	2.81, 3.93
100	20.0	17.5, 22.6	9.48	7.64, 11.3	10.1	7.53, 12.7
200	32.7	28.8, 36.6	17.2	13.4, 20.9	15.1	12.4, 17.6

Extended data table 4.3.1.2 | *B. terrestris* dosage statistics for 24 h and 3 day pre-exposure to nicotine in 0.5 M sucrose. Data correspond to figure 4.3.1 and dose table 4.3.1. Welch's ANOVA for **(a)** the average dose consumed across the 3 day pre-exposure period **(b)** the unexposed 24 h two-way choice data, and **(c)** the 3 day pre-exposed 24 h two-way choice periods. *Post hoc* values are all possible pairwise comparisons between each concentration, Games-Howell adjusted. **(d)** GzLM for the chosen dose during the 24 h two-way choice period between unexposed and pre-exposed bees. **(e)** GzLM for the average dose consumed during the 3 day pre-exposure period against the dose chosen following the pre-exposure period. *Post hoc* values are comparisons between the pre-exposure period and 24 h two-way choice period for each concentration.

(a) $F_{(4)} = 101$ $\eta_p^2 = 0.680$ $p = <0.001$. All *post hoc* value comparisons $p = <0.001$.

(b) $F_{(4)} = 65.7$ $\eta_p^2 = 0.762$ $p = <0.001$. 100 μM vs 200 μM $p = 0.090$. All other *post hoc* value comparisons $p = <0.001$.

†(c) $F_{(4)} = 83.5$ $\eta_p^2 = 0.580$ $p = <0.001$. 6.25 μM vs 12 μM $p = 0.004$, 12 μM vs 25 μM $p = 0.001$, 100 μM vs 200 μM $p = 0.020$. All other *post hoc* value comparisons $p = <0.001$.

(d) Concentration: $\chi^2_{(41} = 805$ $p = <0.001$

Treatment: $\chi^2_{(1)} = 5.73$ $p = 0.017$

Concentration x treatment: $\chi^2_{(4)} = 5.67$ $p = 0.226$

Post hoc comparisons between concentrations (Holm-Bonferroni):

All concentrations are significant at $p = <0.001$

(e) Concentration: $\chi^2_{(4)} = 3.34$ $p = 0.503$

Time: $\chi^2_{(1)} = 88.2$ $p = <0.001$

Concentration x Time: $\chi^2_{(4)} = 1171$ $p = <0.001$

Post hoc comparisons between concentrations at each time point (Holm-Bonferroni): 6.25 μM $p = <0.001$, 12 μM $p = 0.001$, 25 μM $p = 0.001$, 100 μM $p = 0.001$, 200 μM $p = 0.008$

† Data was square-root transformed

4.3.2 The effect of increasing the sucrose concentration to 1.0 M on the bumblebee's preference for nicotine

Bumblebees pre-exposed to nicotine for a period of 3 days varied their total consumption across time depending on the concentration of nicotine they consumed (Fig. 4.3.2a, RM-GLM, time x concentration, Time: $F_{(2.76, 215)} = 3.63$, $\eta_p^2 = 0.044$, $p = 0.016$. Extended data table 4.3.2.1a). Although there was a general trend for increased total consumption in bees that were exposed to nicotine solutions, bees that were provided with 1000 μM nicotine were shown to consume a significantly lower total volume of food during the choice day (*post hoc* LSD, $p = 0.03$).

The data for the 24 h two-way choice test shows that bumblebees preferred 100 μM nicotine over sucrose alone following a period of 3 days pre-exposure to the compound (Fig. 4.3.2b) (GLM, treatment main effect $F_{(1, 172)} = 9.807$, $\eta_p^2 = 0.054$, $p = 0.002$. One-sample T-tests against 0 for 100 μM nicotine (Holm-Bonferroni adjusted), $t_{(22)} = 3.72$ $p = 0.011$).

Bumblebees feeding on 500 μM or 1000 μM nicotine in 1.0 M sucrose consumed less food during the 24 h choice period than bumblebees that were provided with sucrose alone (0 μM) (Fig 4.3.2c) (GLM, main effect of concentration, $F_{(4, 172)} = 3.70$, $\eta_p^2 = 0.079$, $p = 0.001$. *Post hoc* LSD between concentrations, 0 μM vs 500 μM, p = 0.071 (note marginal *p* value), 0 μM vs 1000 μM $p = 0.03$). No other nicotine concentration lead to a decrease in the total volume of solution consumed in comparison to the 0 μM control group (Extended data table. 4.3.2.1c).

The dose of nicotine consumed during the pre-exposure period was as much as 3-30x greater than the lowest nicotine concentration, depending on the treatment (Welch's ANOVA, $F_{(3, 25.3)} = 54.1$ $\eta_p^2 = 0.737$ $p = <0.001$. Extended data table 4.3.2.1). For example, bumblebees provided with 25 μM nicotine consumed a dose of 3.37 μg/bee/day, 95 % CI [2.79, 3.95] whereas those provided with 1000 μM solutions consumed an average dose of 104 μg/bee/day, 95 % CI [80.4, 126]. During the choice period, the pre-exposed bumblebees

Bumblebees: Three day pre-exposure to nicotine in 1.0 M sucrose

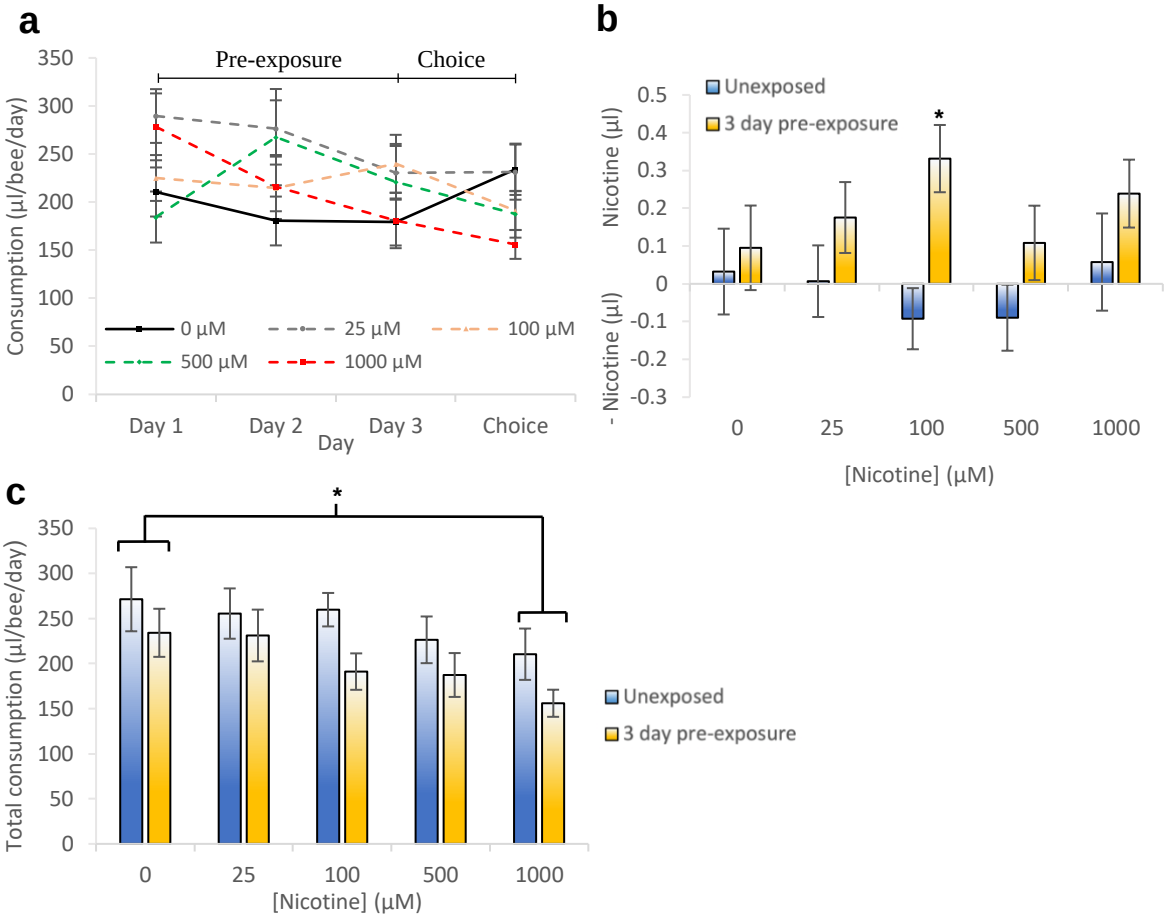

Figure 4.3.2 | Bumblebees display a preference for 100 µM nicotine following 3 days pre-exposure in 1.0 M sucrose. (a) Bumblebees pre-exposed to nicotine for a period of 3 days varied their total consumption as a function of nicotine concentration (Extended data table 4.3.2.1a) **(b)** Bumblebees displayed a preference for 100 µM nicotine only following 3 days pre-exposure (Extended data table 4.3.2.1b). Data represent the mean difference in the amount consumed over the 24 h period. Positive values indicate a preference for nicotine and negative avoidance of nicotine. Asterisks indicate significant differences for one sample T-tests against 0 (Holm-Bonferroni adjusted) (* p <0.05). **(c)** Bumblebees that consumed 1000 µM nicotine during the choice day consumed a significantly lower total volume of solution in comparison to control bees (0 µM) (Extended data table 4.3.2.1c) (LSD adjusted) (*p <0.05). Bars and line graphs indicate mean (±s.e.m.) of consumption (µl) per bee, controlled for by evaporation.

consumed a similar dose to unexposed bees (GzLM, Concentration x treatment: $\chi^2_{(3)}$ = 0.653 p = 0.884). For example, unexposed bees chose to consume a dose of 6.50 μg/bee/day, 95 % CI [5.16, 7.84] during the 24 h two-way choice, whereas pre-exposed bees chose a dose of 7.02 μg/bee/day, 95 % CI [5.36, 8.68]. In all cases, the bees consumed less nicotine on the choice day than they had consumed during the 3 day pre-exposure period (Dose table 4.3.2) (GzLM, main effect of Treatment: $\chi^2_{(1)}$ = 45.6 p = <0.001). For example, bees pre-exposed to 100 μM nicotine consumed 11.3 μg/bee/day, 95 % CI [9.26, 13.3] during the pre-exposure period, but during the test they chose to consume a dose of 7.02 μg/bee/day, 95 % CI [5.36, 8.68].

No unexposed bees died during the 24 h two-way choice period, and mortality was not significantly different to controls in the pre-exposed group (lreg, $\chi^2_{(4)}$ = 5.17, p = 0.270).

Extended data table 4.3.2.1 | *B. terrestris* statistics for 3 day pre-exposure and 24 h two-way choice data in 1.0 M sucrose. Data correspond to figure 4.3.2a-c. **(a)** RM-GLM for the total consumed across the 3 day pre-exposure and 24 h two-way choice period. n: 0 μM (17), 25 μM (17), 100 μM (21), 500 μM (15), 1000 μM (13). **(b)** GLM for the indexed 24 h two-way choice day for unexposed and pre-exposed honeybees. n: 3 day pre-exposed bees, 0 μM (21), 25 μM (21), 100 μM (23), 500 μM (17) 1000 μM (17). Unexposed, 0 μM (9), 25 μM (16), 100 μM (21), 500 μM (17), 1000 μM (20). **(c)** GLM for the total consumed during the 24 h two-way choice period for unexposed and pre-exposed bumblebees and one-way ANOVAs for each treatment.

(a) †	Time: $F_{(2.76, 215)} = 3.63$, $\eta_p^2 = 0.044$, $p = 0.016$ Concentration: $F_{(4, 78)} = 0.926$, $\eta_p^2 = 0.045$, $p = 0.453$ Time x Concentration: $F_{(11, 215)} = 2.73$, $\eta_p^2 = 0.123$, $p = 0.003$ Post hoc (LSD) pairwise comparisons between time. Only significant effects are reported: Day 1 vs choice day $p = 0.006$, day 2 vs choice day $p = 0.019$. *Post hoc* (LSD) comparing nicotine treatments against control at each time point. Only significant effects are reported: Day 1: 0 μM vs 25 μM $p = 0.042$, 48 h 0 μM vs 25 μM $p = 0.04$. Choice day: 0 μM vs 1000 μM $p = 0.03$.
(b)	Treatment: $F_{(1, 172)} = 9.807$, $\eta_p^2 = 0.054$, $p = 0.002$ Concentration: $F_{(4, 172)} = 0.540$, $\eta_p^2 = 0.012$, $p = 0.707$ Treatment x Concentration: $F_{(4, 172)} = 0.876$, $\eta_p^2 = 0.020$, $p = 0.479$ One sample T-test against 0 (Holm-Bonferroni): Unexposed: 0 μM $T_{(8)} = 0.285$ $p = 0783$, 25 μM $T_{(15)} = 0.074$ $p = 1.0$, 100 μM $T_{(20)} = -1.14$ $p = 1.0$, 500 μM $T_{(16)} = -1.03$ $p = 1.0$, 1000 μM $T_{(19)} = 0.448$ $p = 1.0$. 3 day pre-exposure: 0 μM $T_{(20)} = 0.851$ $p = 1.0$, 25 μM $T_{(20)} = 1.87$ $p = 0.608$, 100 μM $T_{(22)} = 3.72$ $p = 0.011$, 500 μM $T_{(16)} = 1.10$ $p = 1.0$, 1000 μM $T_{(16)} = 2.66$ $p = 0.153$.
(c)	Treatment: $F_{(1, 172)} = 10.5$, $\eta p2 = 0.058$, $p = 0.007$ Concentration: $F_{(4, 172)} = 3.70$, $\eta_p^2 = 0.079$, $p = 0.001$ Treatment x Concentration: $F_{(4, 172)} = 0.362$, $\eta_p^2 = 0.008$, $p = 0.835$ *Post hoc* comparisons between concentrations (LSD). Only significant or marginally significant effects are reported: 0 μM vs 1000 μM $p = 0.03$, 0 μM vs 500 μM $p = 0.071$, 25 μM vs 1000 μM $p = 0.013$, 100 μM vs 1000 μM $p = <0.001$, 100 μM vs 500 μM $p = 0.068$ One-way ANOVA for unexposed bees: $F_{(4, 78)} = 1.54$ $\eta_p^2 = 0.073$ $p = 0.199$ One-way ANOVA for 3 day pre-exposed bees: $F_{(4, 78)} = 1.70$ $\eta_p^2 = 0.80$ $p = 0.158$

† Mauchly's test indicated that the assumption of sphericity has been violated ($\chi^2_{(5)} = 22.619$, $p = <0.001$), therefore Huynh-Feldt corrected tests are reported ($\varepsilon = 0.920$).

Dose table 4.3.2 | Summary of the average dose consumed by *B. terrestris* following consumption of nicotine in the 24 h two-way choice periods and during the pre-exposure period. Data correspond to figure 4.3.2a-c. Values represent the average nicotine dose consumed per individual bumblebee (µg/bee/day) and their respective 95 % CIs. Green indicates the dose where a preference is observed.

[Nicotine] µM	Pre-exposure period dose		Chosen dose			
			Pre-exposed		Unexposed	
	µg/bee/day	95 % CI	µg/bee/day	95 % CI	µg/bee/day	95 % CI
25	3.37	2.79, 3.95	1.68	1.41, 1.96	1.69	1.20, 2.19
100	11.3	9.26, 13.3	**7.02**	5.36, 8.68	6.50	5.16, 7.84
500	53.6	39.1, 68.0	26.4	17.3, 35.5	27.0	18.9, 35.0
1000	104	80.4, 126	41.5	30.7, 52.2	48.1	34.0, 62.2

Extended data table 4.3.2.2 | *B. terrestris* dosage statistics for 24 h and 3 day pre-exposure to nicotine in 1.0 M sucrose. Data correspond to figure 4.3.2 and dose table 4.3.2. Welch's ANOVA for **(a)** the average dose consumed across the 3 day pre-exposure period **(b)** the unexposed 24 h two-way choice data, and **(c)** the 3 day pre-exposed 24 h two-way choice periods. *Post hoc* values are all possible pairwise comparisons between each concentration, Games-Howell *post hoc*. **(d)** GzLM for the chosen dose during the 24 h two-way choice period between unexposed bees and bees that had been pre-exposed to nicotine for 3 days prior to the choice. *Post hoc* values are comparisons between each treatment schedule at each concentration (LSD). **(e)** GzLM for the average dose consumed during the 3 day pre-exposure period against the dose chosen following the pre-exposure period. *Post hoc* values are comparisons between the prior period and the chosen period for each concentration.

(a) $F_{(3, 25.3)} = 54.1$ $\eta_p^2 = 0.737$ $p = <0.001$. 500 µM vs 1000 µM $p = 0.009$. All other *post hoc* value comparisons $p = <0.001$.

(b) $F_{(3, 32.3)} = 38.4$ $\eta_p^2 = 0.511$ $p = <0.001$. 500 µM vs 1000 µM $p = 0.073$. All other *post hoc* value comparisons $p = <0.001$.

(c) $F_{(3, 30.5)} = 36.4$ $\eta_p^2 = 0.564$ $p = <0.001$. 100 µM vs 500 µM $p = 0.004$, 500 µM vs 1000 µM $p = 0.176$. All other *post hoc* value comparisons $p = <0.001$.

(d) Concentration: $\chi^2_{(31} = 508$ $p = <0.001$

Treatment: $\chi^2_{(3)} = 0.031$ $p = 0.860$

Concentration x treatment: $\chi^2_{(3)} = 0.653$ $p = 0.884$

Post hoc comparisons between treatments at each concentration (LSD):

25 µM $p = 1.000$, 100 µM $p = 1.000$, 500 µM $p = 1.000$, 1000 µM $p = 1.000$

(e) Concentration: $\chi^2_{(3)} = 617$ $p = <0.001$

Treatment: $\chi^2_{(1)} = 45.6$ $p = <0.001$

Concentration x treatment: $\chi^2_{(3)} = 2.65$ $p = 0.449$

Post hoc comparisons between concentrations (Holm-Bonferroni): All pairwise comparisons < 0.001.

4.3.3 The effect of increasing the length of pre-exposure on the bumblebee's preference for 100 μM nicotine in 1.0 M sucrose

Bumblebees consumed equivalent volumes of solution regardless of whether they were pre-exposed to nicotine or were presented with sucrose alone (Extended data table 4.3.3.1a). In both treatments, bees were seen to decrease the total amount of solution they consumed over the course of the experiment (Fig. 4.3.3a, RM-GLM, main effect of Time: $F_{(4.53, 235)} = 8.50$, $\eta_p^2 = 0.141$, $p = <0.001$. Extended data table 4.3.3.1a).

These data were compared to both the unexposed 24 h two-way choice and 3 day pre-exposed 24 h two-way choice with 100 μM nicotine data from the sections above. There was a marginal effect of treatment on the preference for nicotine (Fig 4.3.3b GzLM, main effect of treatment, $\chi^2_{(2)} = 5.82$ $p = 0.054$). *Post hoc* analysis revealed that both the 3 day and 5 day treatments were significantly different from unexposed bees during the 24 h two-way choice (Independent samples T-tests, two-tailed, LSD: $T_{(72)} = -2.65$ $p = 0.009$ and $T_{(83)} = -2.40$ $p = 0.019$, respectively). Increasing the length of pre-exposure to 5 days did not result in a stronger preference for nicotine ($T_{(97)} = 0.680$ $p = 0.498$). One-sample T-tests against 0 indicate that bees pre-exposed to nicotine for a period of 5 days prefer 100 μM nicotine over sucrose (Fig 3.4b. $T_{(34)} = 3.26$ $p = 0.015$, Holm-Bonferroni corrected).

The total volume consumed by bumblebees over the choice period depended on the treatment (Fig 4.3.3c. GLM, main effect of treatment: $F_{(2, 123)} = 17.8$, $\eta p2 = 0.225$, $p = <0.001$). However, total consumption for nicotine-exposed bees in each treatment group was not significantly different to their respective sucrose-only controls (Extended data table 4.3.3.1c)

Bees pre-exposed to nicotine for a period of 5 days on average consumed a greater daily dose of nicotine than bees pre-exposed to 100 μM nicotine over the 3 day pre-exposure period (GLM Pre-exposure Treatment: $F_{(1, 109)} = 34.7$, $\eta p2 = 0.241$, $p = <0.001$. *Post hoc* (LSD) 5 day pre-treatment vs 3 day pre-treatment $p = <0.001$). Bees in the 5 day pre-exposure consumed on average 18.7 μg/bee/day, 95 % CI [17.0, 20.4], whereas bees pre-exposed for 3 days consumed on average 11.3 μg/bee/day 95 % CI [9.28, 13.3]. Both the 3 day and 5 day pre-exposed bees chose to consume a significantly lower dose over the

Bumblebees: Three and five day pre-exposure to 100 µM nicotine in 1.0 M sucrose

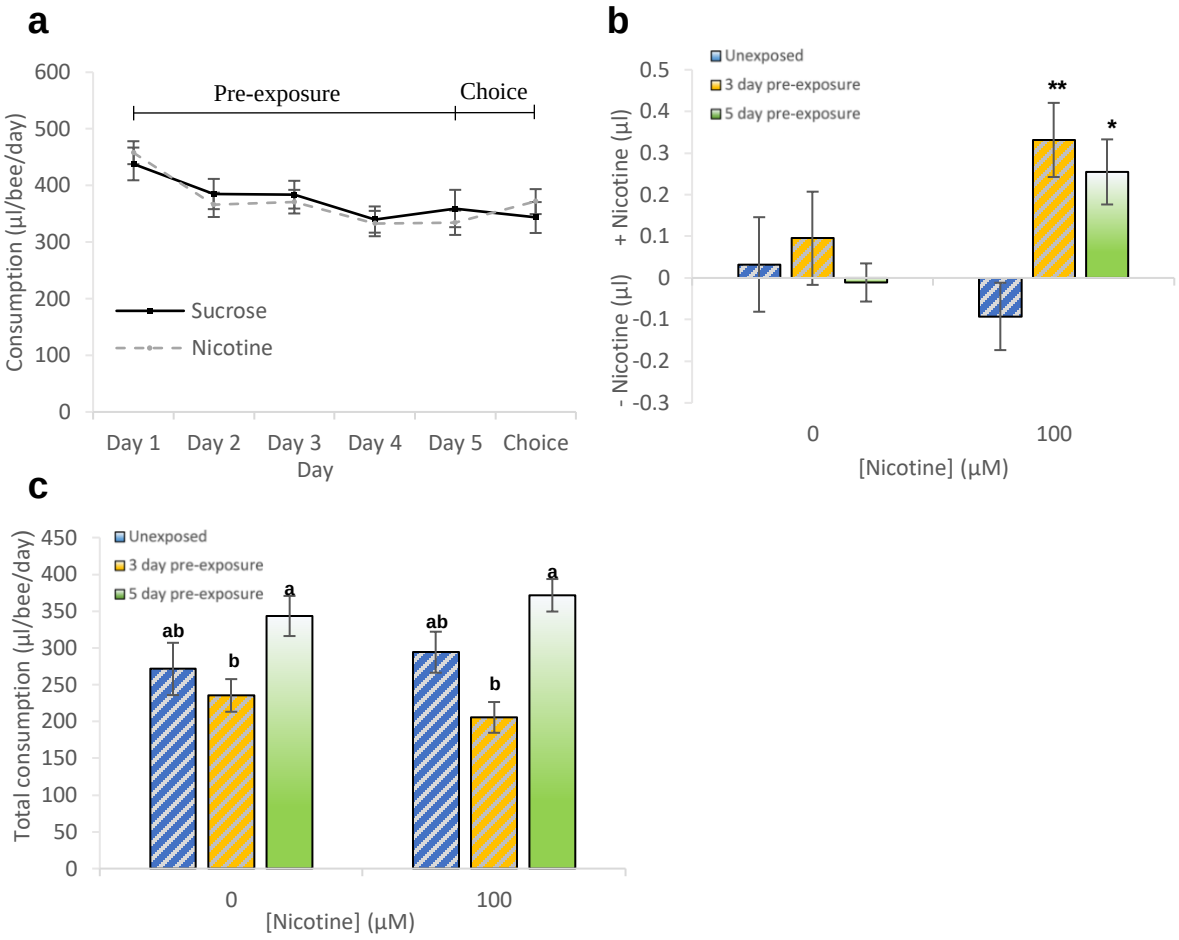

Figure 4.3.3 | Bumblebees did not display a greater preference for 100 µM nicotine following a prolonged period of pre-exposure (a) Bumblebee pre-exposed to nicotine for a period of 5 days varied the total volume they consumed across time (Extended data table 4.3.3.1a). **(b)** Bumblebees displayed a preference for 100 µM nicotine following 5 days pre-exposure; however, this preference was not significantly different to bees pre-exposed to nicotine for a period of 3 days (Extended data table 4.3.3.1b). Data represent the mean difference in the amount consumed over the 24 h period. Positive values indicate a preference for nicotine and negative avoidance of nicotine. Dashed lines represent data obtained for 0 µM and 100 µM 3 day pre-exposed bumblebees collected in section 4.3.2, used for comparison here. Asterisks indicate significant differences for one sample T-tests against 0 (*p <0.05; **p <0.01). **(c)** Bumblebees varied the total volume consumed over the choice period depending on the treatment they received. However, total consumption for nicotine-exposed bees in each treatment group was not significantly different to their specific controls (Extended data table 4.3.3.1c). Dashed lines represent data obtained for 0 µM and 100 µM 3 day pre-exposed bumblebees and unexposed bumblebees collected in section 4.3.2, used for comparison here. Bars and line graphs indicate mean (±s.e.m.) of consumption (µl) per bee, controlled for by evaporation. Different letters indicate statistically significant differences between groups (*Post hoc* LSD).

24 h two-way choice period than what they consumed during the pre-exposure period (Time: $F_{(1, 109)} = 34.6$, $\eta p2 = 0.241$, $p = <0.001$. Extended data table 4.3.3.2b).

During the 24 h choice test bees pre-exposed to nicotine for 3 days consumed a similar dose to unexposed bees (One-Way ANOVA, $F_{(2, 78)} = 8.73$, $p = <0.001$. *Post hoc* LSD, unexposed choice vs 3 day pre-exposed bees $p = 0.823$). For example, unexposed bees consumed a dose of 6.50 μg/bee/day, 95 % CI [5.16, 7.84], whereas 3 day pre-exposed bees consumed a dose of 7.02 μg/bee/day 95 % CI [5.36, 8.68]. In contrast, bees pre-exposed to nicotine for a period of 5 days consumed a larger dose of 11.7 μg/bee/day, 95 % CI [8.46, 14.9], (One-Way ANOVA, $F_{(2, 78)} = 8.73$, $p = <0.001$. *Post hoc* (LSD) unexposed choice vs 5 day pre-exposed bees $p = <0.001$, 3 day pre-exposed vs 5 day pre-exposed bees $p = 0.001$).

No bees died in either the control or nicotine treatment groups over the course of the experiment.

Extended data table 4.3.3.1 | *B. terrestris* statistics for unexposed, 3 and 5 day pre-exposure and 24 h two-way choice data in 1.0 M sucrose. Data correspond to figure 4.3.3a-c. **(a)** RM-GLM for the total consumed across the 5 day pre-exposure and 24 h two-way choice period. n: 0 μM = 22, 100 μM = 36 **(b)** GzLM for the indexed 24 h two-way choice day for unexposed and pre-exposed bumblebees. *Post hoc* tests represent independent samples T-tests between treatments. n: unexposed bees, 0 μM = 9, 100 μM = 21. 3 day pre-exposed bees, 0 μM = 21, 100 μM = 23. 5 day pre-exposed bees, 0 μM = 22, 100 μM = 37. **(c)** GLM for the total consumed during the 24 h two-way choice period for unexposed and pre-exposed bumblebees, and independent samples T-test or Mann-Whitney U-tests for the total consumed during the 24 h two-way choice period for unexposed and pre-exposed bumblebees.

(a) †	Time: $F_{(4.53, 235)} = 8.50$, $\eta_p^2 = 0.141$, $p = <0.001$ Concentration: $F_{(1, 52)} = 0.004$, $\eta_p^2 = <0.001$, $p = 0.949$ Time x Concentration: $F_{(4.53, 235)} = 0.684$, $\eta_p^2 = 0.013$, $p = 0.621$ *Post hoc* pairwise comparisons for time (LSD): Day 1 vs choice day $p = <0.001$. All other comparisons $p = >0.05$.
(b)	Concentration: $\chi^2_{(1)} = 3.66$ $p = 0.103$ Treatment: $\chi^2_{(2)} = 5.82$ $p = 0.054$ Concentration x treatment: $\chi^2_{(2)} = 4.37$ $p = 0.113$. *Post hoc* analysis of treatment (Independent samples T-test, significance 2 tailed) (LSD): Unexposed versus 3 day pre-exposure $T_{(72)} = -2.65$ $p = 0.009$, Unexposed vs 5 day pre-exposure $T_{(83)} = -2.40$ $p = 0.019$, 3 day pre-exposure versus 5 day pre-exposure $T_{(97)} = 0.680$ $p = 0.498$ One sample T-test against 0 (Holm-Bonferroni): Unexposed 0 μM $T_{(8)} = 0.285$ $p = 1.000$, unexposed 100 μM $T_{(20)} = -1.14$ $p = 1.000$, 3 day 0 μM $T_{(20)} = 0.851$ $p = 1.000$, 3 day 100 μM $T_{(22)} = 3.72$ $p = 0.006$, 5 day 0 μM $T_{(19)} = -2.37$ $p = 1.000$, 5 day 100 μM $T_{(34)} = 3.26$ $p = 0.015$
(c) ††	Treatment: $F_{(2, 123)} = 17.8$, $\eta p2 = 0.225$, $p = <0.001$ Concentration: $F_{(1, 123)} = 0.018$, $\eta_p^2 = <0.001$, $p = 0.895$ Treatment x Concentration: $F_{(2, 123)} = 1.07$, $\eta_p^2 = 0.017$, $p = 0.345$ *Post hoc* analysis of treatment x concentration (LSD): Unexposed 0 μM vs 3 day pre-exposed 0 μM $p = 0.450$, unexposed 0 μM vs 5 day pre-exposed $p = 0.108$, 0 μM pre-exposed vs 5 day pre-exposed $p = 0.003$, Unexposed 100 μM vs 3 day pre-exposed 100 μM $p = 0.005$, unexposed 100 μM vs 5 day pre-exposed $p = 0.022$, 100 μM pre-exposed vs 5 day pre-exposed $p = <0.001$. Independent samples T-test for the unexposed data: $T_{(28)} = -5.12$ $p = 0.613$ Mann-Whitney U-Test for the 3 day pre-exposed data: U = 195, $p = 0.275$ Independent samples T-test for the 5 day pre-exposed data: $T_{(53)} = -0.742$ $p = 0.461$

† Mauchly's test indicated that the assumption of sphericity has been violated ($\chi^2_{(5)} = 29.9$, $p = 0.008$), therefore Huynh-Feldt corrected tests are reported ($\varepsilon = 0.906$)

†† Data was square-root transformed

Dose table 4.3.3 | Summary of the average dose consumed by *B. terrestris* following consumption of nicotine in the 24 h two-way choice periods and during the 3 and 5 day pre-exposure period. Data correspond to figure 4.3.3a-c. Values represent the average nicotine dose consumed per individual bumblebee (µg/bee/day) and their respective 95 % CIs. Green indicates the dose where preference is observed.

Treatment	Chosen dose	
	µg/bee/day	95 % CI
Unexposed choice dose	6.50	5.16, 7.84
Average dose across the 3 day pre-exposure	11.3	9.28, 13.3
Choice dose following 3 days pre-exposure	**7.02**	5.36, 8.68
Average dose across the 5 day pre-exposure	18.7	17.0, 20.4
Choice dose following 5 days pre-exposure	**11.7**	8.46, 14.9

Extended data table 4.3.3.2 | *B. terrestris* dosage statistics for unexposed 24 h, 3 day, and 5 day pre-exposure to nicotine in 1.0 M sucrose. Data correspond to figure 4.3.3 and dose table 4.3.3. **(a)** GLM comparing the average dose consumed over the 3 and 5 day pre-exposure period to the chosen dose over the 24 h two-way choice period for 3 and 5 day pre-exposed bees **(b)** One-Way ANOVA comparing the dose chosen over the 24 h two-way choice period for the unexposed, 3 day pre-exposed, and 5 day pre-exposed bees.

†(a) Pre-exposure Treatment: $F_{(1, 109)} = 34.7$, $\eta p2 = 0.241$, $p = <0.001$

Time: $F_{(1, 109)} = 34.6$, $\eta p2 = 0.241$, $p = <0.001$

Time x Pre-exposure Treatment: $F_{(1, 109)} = 0.621$, $\eta_p^2 = 0.006$, $p = 0.432$

Post hoc (LSD): 5 day pre-exposure vs 5 day choice $p = <0.001$, 3 day pre-exposure vs 3 day choice $p = 0.002$, 5 day pre-treatment vs 3 day pre-treatment $p = <0.001$, 5 day chosen dose vs 3 day chosen dose $p = <0.001$.

(b) One-Way ANOVA: $F_{(2, 78)} = 8.73$, $p = <0.001$. *Post hoc* (LSD): unexposed vs 3 day $p = 0.823$, unexposed vs 5 day $p = <0.001$, 3 day vs 5 day $p = 0.001$.

† Data was square-root transformed

4.3.4 The bumblebee's response to nicotine when offered a choice between the compound and a higher molarity sucrose solution

If nicotine is acting as an addictive agent in the bee, it was expected that pre-exposure to nicotine would result in devaluation of a higher molarity sucrose solution during the 24 h choice. To test whether nicotine pre-exposure affects the relative evaluation of a sucrose reward, bumblebees were pre-exposed to 0 μM (control) or 100 μM nicotine for a period of 3 days. During a 24 h choice assay, each bumblebee was assigned to one of the following groups: 100 μM nicotine in 1.0 M sucrose, 1.0 M sucrose or 1.0 M, 1.1 M, 1.2 M, or 1.3 M sucrose.

Bees pre-exposed to nicotine for a period of 3 days consumed a significantly greater total volume during the first day of pre-exposure in comparison to control bees (Fig. 4.3.4a) (RM-GLM, Time x Treatment: $F_{(2, 546)} = 7.89$, $\eta_p^2 = 0.028$, $p = <0.001$. *Post hoc* comparisons between treatments during the initial 24 h period concentration (LSD) $p = 0.007$). On average, nicotine-exposed bumblebees consumed 31.7 μl more than control bees over the initial day of pre-exposure (Extended data table 4.3.4.1c). However, total consumption was not significantly different to control over the remaining 2 days (Fig. 4.3.4a. Extended data table 4.3.4.1c).

The bumblebees preference for the higher molarity sucrose in both the nicotine pre-exposed and sucrose pre-exposed bees varied as a function of pre-exposure and sucrose molarity (GLM, Pre-exposure treatment x Sucrose concentration: $F_{(3, 270)} = 3.46$, $\eta_p^2 = 0.037$, $p = 0.017$). The data for the 24 h two-way choice test showed that similar to previous experiments (Fig 4.3.3b) bumblebees displayed a preference for 100 μM nicotine administered in 1.0 M sucrose in comparison to 1.0 M sucrose alone (Extended data table 4.3.4.1c). Both the sucrose pre-exposed and nicotine pre-exposed bees displayed a significant preference for 1.2 and 1.3 M sucrose over 1.0 M sucrose or 100 μM nicotine, respectively (Fig 4.3.4b. Extended data table 4.3.4.1b). Whereas no preference was observed for 1.1 M sucrose in either treatment group (Fig 3.4.4b, extended data table 3.4.4.1b).

Bumblebees: Valence experiment

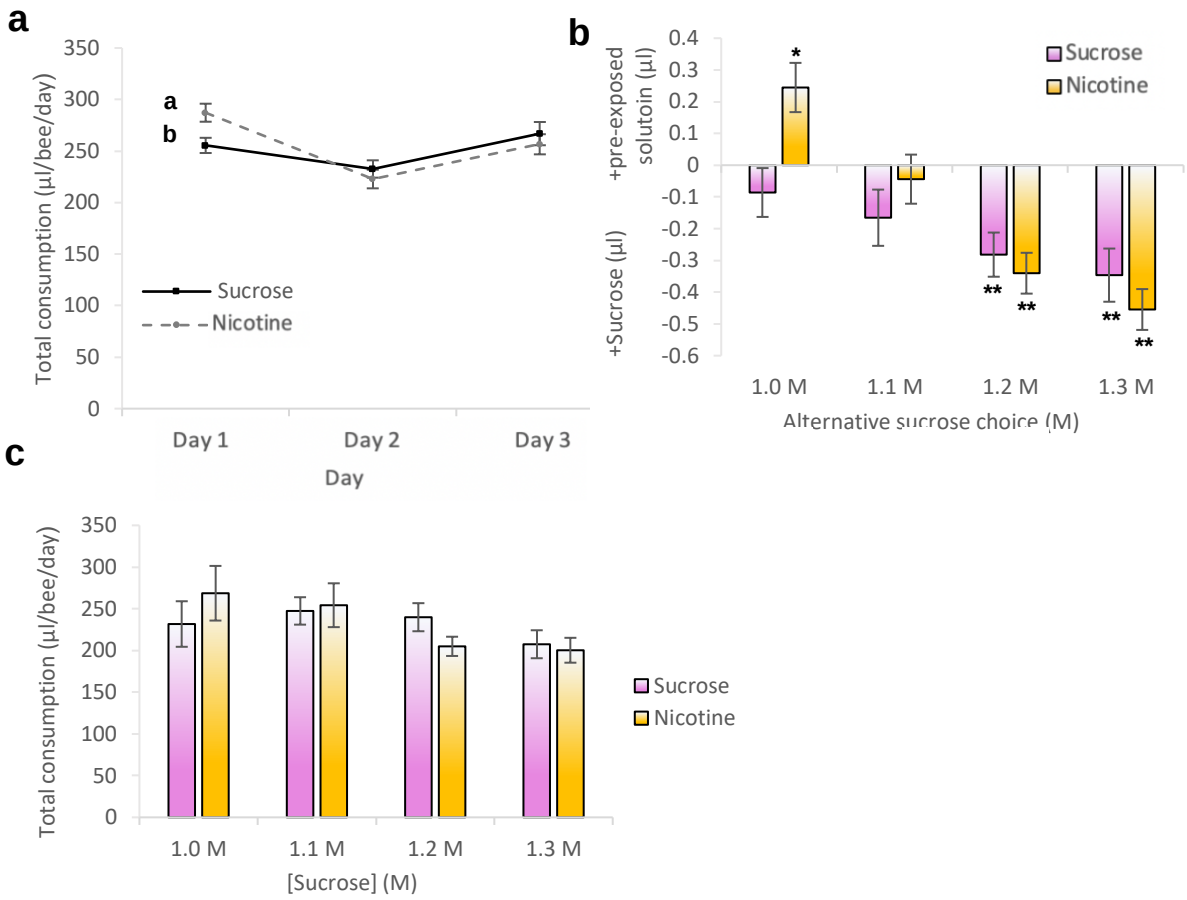

Figure 4.3.4 | Bumblebees did not display a preference for 100 µM nicotine when offered a choice between nicotine or a higher molarity sucrose solution. (a) Bees pre-exposed to nicotine for a period of 3 days consumed a significantly greater total volume over the first day of pre-exposure in comparison to control bees (Extended data table 4.3.4.1a) **(b)** Preferences for nicotine or for higher molarity sucrose varied as a function of pre-exposure treatment and sucrose concentration (Extended data table 4.3.4.1b). Bumblebees exhibited a preference for nicotine only during a two-way choice between 100 µM nicotine in 1.0 M sucrose or 1.0 M sucrose alone (Extended data table 4.3.4.1b). Both sucrose pre-exposed and nicotine pre-exposed bees were indifferent to 1.1 M sucrose, whereas they displayed a significant preference for the higher molarity sucrose concentrations (Extended data table 4.3.4.1b). Pink bars represent bees that were fed 1.0 M sucrose for 3 days followed by a choice between 1.0 M sucrose or a higher molarity sucrose of varying concentrations. Yellow bars represent bees that received 3 days pre-exposure to nicotine followed by a choice between 100 µM nicotine in 1.0 M sucrose or sucrose of varying concentrations. Data represent the mean difference in the amount consumed over the 24 h period. Positive values indicate a preference for the pre-exposed solution (i.e. Pink bars: a preference for 1.0 M sucrose alone. Yellow bars: a preference for 100 µM nicotine in 1.0 M sucrose), and negative a preference for sucrose. **(c)** Total consumption across the 24 h two-way choice period did not vary as a function of either sucrose concentration or pre-treatment to nicotine or sucrose (Extended data table 4.3.4.1c). Bars and line graphs indicate mean (±s.e.m.) of consumption (µl) per bee, controlled for by evaporation.

Bumblebees consumed a similar total volume of food over the choice day irrespective of both their pre-exposure period and the magnitude of the alternative reinforcer (GzLM, Sucrose concentration x pre-treatment: $\chi^2_{(2)} = 4.37$ $p = 0.113$. Extended data table 4.3.4.1c).

Nicotine pre-exposed bees consumed a similar nicotine dose across the 3 day pre-exposure period (Dose table 4.3.4) (One-Way ANOVA: $F_{(3, 137)} = 0.242$ $p = 0.867$), with all doses consumed in the range of 12.3-13.1 μg/bee/day. During the 24 h two-way choice period, the nicotine dose depended on both the treatment and pre-exposure period (GzLM Sucrose concentration x exposure: $\chi^2_{(3)} = 35.4$ $p = <0.001$). In all cases, bumblebees consumed a significantly lower dose over the 24 h two-way choice period (Extended data table 4.3.4.2a). For example, bees in the 1.0 M choice group consumed a dose of 12.6 μg/bee/day, 95 % CI [10.7, 14.5] during pre-exposure but chose a dose of 8.24 μg/bee/day, 95 % CI [6.18, 10.3] during the test.

The bumblebees chosen dose decreased as the sucrose molarity increased (Extended data table 4.3.4.2b). Bumblebees choosing between 1.0 M sucrose and 1.0 M sucrose with nicotine consumed an average dose of 8.24 μg/bee/day, 95 % CI [6.18, 10.3]. Whereas, bees offered a choice between 100 μM nicotine or 1.1, 1.2, or 1.3 M sucrose consumed a dose of 6.55 μg/bee/day, 95 % CI [4.58, 8.52], 3.45 μg/bee/day, 95 % CI [2.63, 4.28], and 2.87 μg/bee/day, 95 % CI [1.81, 3.93], respectively, with only the two highest sucrose molarities reaching significance (*post hoc* Holm-Bonferroni, $p = 0.409$, $p = 0.017$ and 0.009, respectively).

Only a single bee died across the course of the experiment in the nicotine pre-exposed vs 1.2 M sucrose treatment group.

Extended data table 4.3.4.1 | *B. terrestris* statistics for the higher molarity choice. Data correspond to figure 4.3.4a-c. **(a)** RM-GLM comparing the pooled 3 day pre-exposure period for sucrose and nicotine treatments. Sucrose pre-exposed n = 134, nicotine pre-exposed n = 142 **(b)** GLM comparing the indexed two-way choice period and One-sample T-tests against 0 n: Sucrose prior: 1.0 M (33), 1.1 M (33), 1.2 M (36), 1.3 M (33), nicotine prior: 1.0 M (35), 1.1 M (36), 1.2 M (36), 1.3 M (36). **(c)** GzLM comparing the total consumption over the 24 h two-way choice period and one-way ANOVA or Kruskal Wallis test for each treatment.

(a) †	Time: $F_{(2, 546)} = 33.3$, $\eta p2 = 0.109$, $p = <0.001$ Treatment: $F_{(1, 273)} = 0.050$, $\eta_p^2 = <0.001$, $p = 0.823$ Time x Treatment: $F_{(2, 546)} = 7.89$, $\eta_p^2 = 0.028$, $p = <0.001$ *Post hoc* pairwise comparisons for time (LSD): Day 1 vs day 2 $p = <0.001$, day 1 vs day 3 $p = 0.033$, day 2 vs day 3 $p = <0.001$. *Post hoc* comparisons between treatments at each time point (LSD): Day 1 $p = 0.007$, Day 2 $p = 0.322$, Day 3 $p = 0.468$.
(b)	Pre-exposure treatment: $F_{(1, 270)} = 1.80$, $\eta_p^2 = 0.007$, $p = 0.181$ Sucrose concentration: $F_{(3, 270)} = 16.1$, $\eta p2 = 0.152$, $p = <0.001$ Pre-exposure treatment x Sucrose concentration: $F_{(3, 270)} = 3.46$, $\eta_p^2 = 0.037$, $p = 0.017$ *Post hoc* comparisons between treatments at each concentration (Bonferroni): 1.0 M sucrose $p = 0.002$, 1.1 M sucrose $p = 0.257$, 1.2 M sucrose $p = 0.576$, 1.3 M sucrose $p = 0.312$. *Post hoc* comparisons between concentrations (Bonferroni): 1.0 M vs 1.1 M $p = 0.096$, 1.0 M vs 1.2 M $p = <0.001$, 1.0 M vs 1.3 M $p = <0.001$, 1.1 M vs 1.2 M $p = 0.037$, 1.1 M vs 1.3 M $p = <0.001$, 1.2 M vs 1.3 M $p = 1.000$. One sample T-tests against 0 (Holm-Bonferroni): Suc 1.0 M $t_{(32)} = -1.12$ $p = 0.542$, Suc 1.1 M $t_{(32)} = -1.87$ $p = 0.213$, Suc 1.2 M $t_{(35)} = -4.05$ $p = 0.008$, Suc 1.3 M $t_{(32)} = -4.14$ $p = 0.008$, Nic 1.0 M $t_{(34)} = 3.16$ p = 0.012, Nic 1.1 M $t_{(35)}$ -0.569 $p = 0.573$, Nic 1.2 M $t_{(35)} = -5.29$ $p = 0.008$, Nic 1.3 M $t_{(35)} = -7.07$ $p = 0.008$.
(c)	Sucrose concentration: $\chi^2_{(3)} = 3.66$ $p = 0.103$ Pre-treatment: $\chi^2_{(3)} = 7.03$ $p = 0.071$ Sucrose concentration x pre-treatment: $\chi^2_{(2)} = 4.37$ $p = 0.113$ One-way ANOVA for sucrose pre-exposed bees: $F_{(3, 130)} = 1.32$ $\eta_p^2 = 0.030$ $p = 0.270$ Kruskal Wallis test for nicotine pre-exposed bees: $\chi^2_{(3)} = 2.467$, $p = 0.481$

† Data was square-root transformed

Dose table 4.3.4 | Summary of the average dose consumed by *B. terrestris* following consumption of nicotine in the 24 h two-way choice periods and during the pre-exposure period. Data correspond to figure 4.3.4a-c. Values represent the average nicotine dose consumed per individual bumblebee (µg/bee/day) and their respective 95 % CIs. Green indicates the dose where a significant preference for nicotine is observed.

Nicotine in 1.0 M sucrose versus either 1.0 M, 1.1 M, 1.2 M or 1.3 M sucrose	Average dose across the 3 day pre-exposure period		Chosen dose over the 24 h two-way choice period	
	µg/bee/day	95 % CI	µg/bee/day	95 % CI
1.0 M sucrose	12.6	10.7, 14.5	**8.24**	6.18, 10.3
1.1 M sucrose	12.3	11.1, 13.6	6.55	4.58, 8.52
1.2 M sucrose	12.9	11.3, 14.4	3.45	2.63, 4.28
1.3 M sucrose	13.1	11.8, 14.4	2.87	1.81, 3.93

Extended data table 4.3.4.2 | *B. terrestris* dosage statistics for bees offered a higher molarity sucrose choice. Data correspond to figure 4.3.4 and dose table 4.3.4. **(a)** One-way ANOVA comparing the average dose consumed across the pre-exposure period across treatments. **(b)** GzLM comparing the average dose consumed across the pre-exposure period for each treatment against the final chosen dose during the 24 h two-way choice period

(a) One-Way ANOVA: $F_{(3, 137)} = 0.242$ $p = 0.867$

†(b) Sucrose Concentration: $\chi^2_{(3)} = 30.0$ $p = <0.001$

exposure: $\chi^2_{(1)} = 162.8$ $p = <0.001$

Sucrose concentration x exposure: $\chi^2_{(3)} = 35.4$ $p = <0.001$

Post hoc comparisons between treatments (Holm-Bonferroni): 1.0 M vs 1.1 M $p = 0.409$, 1.0 M vs 1.2 M $p = 0.017$, 1.0 M vs 1.3 M $p = 0.009$, 1.1 M vs 1.2 M $p = 0.274$, 1.1 M vs 1.3 M $p = 0.214$, 1.2 M vs 1.3 M $p = 0.813$.

Post hoc comparisons between the pre-exposure dose and the chosen dose (Holm-Bonferroni): 1.0 M pre-exposed vs 1.0 M choice dose, $p = <0.001$, : 1.1 M pre-exposed vs 1.1 M choice dose, $p = <0.001$, : 1.2 M pre-exposed vs 1.2 M choice dose, $p = <0.001$, : 1.3 M pre-exposed vs 1.3 M choice dose, $p = <0.001$.

† Data was square-root transformed.

4.4 Discussion

When unexposed bees were offered nicotine in an immediate 24 h two-way choice, no preference was observed for the compound. However, a significant preference was observed in adult worker bumblebees when they had been pre-exposed to nicotine for 3 or 5 days in a 1.0 M sucrose solution. Despite this, no preference was observed when bees were offered a choice between nicotine and an alternative reward of greater magnitude. These results show that the schedule of drug reinforcement has an effect on whether preferential consumption of nicotine is observed in forager bumblebees.

4.4.1 Bumblebee do not display the characteristic feeding response observed in honeybees over the course of the pre-exposure period

Honeybees in chapter 3.0 were shown to display a characteristic feeding response during the pre-exposure periods. Specifically, honeybees typically consumed a greater total volume of food on the first day of pre-exposure, followed by a rapid decline in feeding on the second day, before consuming approximately equal total volumes of food over the remaining two days of the experiment. As discussed earlier (3.4.1), I hypothesise that this characteristic feeding response arises due to the stress and starvation that honeybees experience during their collection on the first day of experimentation. In contrast to honeybees, bumblebees in this study typically consumed approximately equal volumes of food over the course of the pre-exposure period (exceptions to this are discussed below). Although the results from earlier bumblebee experiments (i.e. pre-exposed for 3 days in 0.5 M sucrose and pre-exposed for 3 days in 1.0 M sucrose) displayed substantial variability across treatments in the total volume of food consumed over the pre-exposure period, in contrast to later experiments (i.e. pre-exposed for 5 days in 1.0 M sucrose and the valence experiment), this is likely explained by the sample size of these experiments. For example, the sample size for bumblebee pre-exposed to nicotine in 1.0 M sucrose for 3 days was n = 17-23, whereas the sample size for bumblebees pre-exposed to nicotine for 5 days, and the sample size for bees in the valence experiment were n = 23-37 and n = 134-142, respectively. Despite this variability, it remained evident that bumblebees do not display the characteristic feeding response observed in the earlier study with honeybees.

The bumblebee colonies used in these experiments were commercial colonies purchased from a supplier. Commercial colonies are housed in a box under constant darkness, and the bumblebees only means of exiting the box is through a shutter door that can be opened or closed by the experimenter. This shutter system allows for rapid bumblebee collection. As bumblebees are phototaxic, they readily move towards sources of light (Morandin et al. 2012). By placing the colonies on a bench in the laboratory near a light source, such as a window, and opening the shutter door, bumblebees will readily exit the colony, where they can be collected in a small phial placed at the shutter entrance. This collection procedure is in stark contrast to that of honeybees, where hives are maintained outside, and weather conditions can dramatically alter the time taken to collect experimental animals, resulting in extended periods of starvation on the initial day of experimentation (discussed in detail in 3.4.1). As it takes considerably less time to collect bumblebees, bumblebees in these experiments were not subjected to the extended periods of starvation, and likely stress, that honeybees underwent on the initial day of experimentation in the previous chapter. This may explain the differences observed in the feeding behaviour between these experiments and the earlier honeybee experiments. Although bumblebees in the valence experiment do show some evidence of the characteristic feeding response observed in honeybee experiments; consuming the greatest total volume on the first day of pre-exposure, followed by a rapid decline in feeding on the second day, before consuming approximately equal total volumes of food over the remaining pre-exposure day, the decline observed on the second day in the sucrose control treatment was only ~ 23 μl, representing a reduction in feeding of 13 % to that of the first day of pre-exposure. In contrast, honeybees that consumed identical concentrations of sucrose were shown to reduce their feeding by 32 μl on the second day of pre-exposure, representing a 37 % reduction in feeding to that of the first day of pre-exposure (see 0 μM control, 3.3.2). Therefore, even though bumblebees may exhibit the characteristic feeding response observed in honeybees in some instances, suggesting that bumblebees may be experiencing some starvation and/or stress over the first day of experimentation, the magnitude of this feeding response is substantially lower than that seen in honeybees, which I hypothesise is due to the differences in bee collection methods.

4.4.2 Bumblebees total consumption varied depending on the sucrose concentration

Previous experiments in bumblebees have shown that bumblebees will consume approximately twice as much of a 0.25 M sucrose solution than a 0.5 M solution in order to reach their carbohydrate needs (Stabler et al. 2015). Therefore, although not directly assessed in this study, it was expected that bumblebees would consume approximately twice as much of a 1.0 M sucrose solution to that of a 0.5 M sucrose solution. Although bumblebees were shown to increase the total volume consumed when presented with a 0.5 M sucrose solution to that of a 1.0 M sucrose solution in some instances, e.g. 3 day pre-exposed bumblebees that were provided with 0.5 M sucrose alone consumed ~328 ul of solution over the first day of pre-exposure, whereas 3 day pre-exposed bumblebees that were provided with 1.0 M sucrose alone consumed ~210 ul of solution over the first day of pre-exposure (representing a decrease of 118 ul), this was not observed in other instances, e.g. 5 day pre-exposed bumblebees that were provided with 1.0 M sucrose alone consumed ~438 ul of solution over the first day of pre-exposure (representing an increase of 110 ul in comparison to the total consumption observed in bumblebees that 0.5 M sucrose). Although this was unexpected, this is likely explained by the fact that bumblebees vary dramatically in size (Goulson et al. 2002), and larger bumblebees are known to consume a greater total volume of a sucrose solution than smaller bees due to their increased energy requirements (Brown and Brown, 2019). This is in contrast to honeybees, which are homogenous in size and typically consume equivalent volumes of food to one another (McCullan and Brown, 2006).

Although all bumblebees used in these assays had a thorax width > 4.5 mm to minimise the likelihood of nurse bee inclusion (see general methods), given that *B. terrestris* foragers can reach a maximum thorax width ~6.8 mm (Goulson et al. 2002), indicates that the bumblebees used in this thesis likely varied in size and therefore varied in the total volume of sucrose that they would consume. Furthermore, not only are there individual differences in the sizes of worker bumblebees within a colony (Goulson et al. 2002), but there are also differences across colonies, and some colonies may produce, on average, larger workers than others (Couvillon et al. 2010). The commercial colonies used in this thesis contain ~50-100 workers, of which approximately half will meet the > 4.5 mm thorax criteria used in this thesis (Goulson et al. 2002), leaving only ~25-50 viable bumblebees from each colony for experimentation. The sample sizes used in this thesis

varied across experiments; however, as an example, collectively, there were 99 bees used in the 1.0 M unexposed assay alone. Therefore, multiple colonies were required to obtain enough experimental animals to complete all experiments in this chapter. As multiple colonies were required, and, due to limitations in the total number of bee cages that can be placed into an incubator at one time (55 cages), it was not possible to run all experiments in parallel; therefore, different colonies were used across experiments. This may account for the variation in the total volume consumed in these experiments from that expected. i.e. if the colonies used for the 1.0 M sucrose 5 day pre-exposure assay had, on average, larger workers than those used for the 0.5 M 3 day pre-exposure assay, then it would be expected that the bumblebees in the 1.0 M 5 day pre-exposure assay would have a greater energy requirement, and therefore may consume a greater total volume of solution than bumblebees in the 0.5 M 3 day pre-exposure assay. Although a cut-off width of > 4.5 mm was used in these assays, unfortunately, individual thorax widths were not recorded; therefore, it would not be possible to directly assess the impact of bumblebee size on the total volume consumed across experiments.

4.4.3 High concentrations of nicotine suppress feeding in the bumblebee

Bumblebees were shown to reduce the total volume of food that they consumed in comparison to the sucrose-only controls on two occasions. Unexposed bumblebees that were provided with 100 μM or 200 μM nicotine dissolved in 0.5 M sucrose reduced their feeding over the 24 h two-way choice period, whereas pre-exposed bumblebees did not. In addition, both unexposed and pre-exposed bumblebees decreased the total volume of food consumed over the 24 h two-way choice period when 500 μM or 1000 μM nicotine (representing a dose of 26.4 and 48.1 μg/bee/day, respectively) were presented in a 1.0 M sucrose solution. This suggests that high concentrations of nicotine suppress feeding behaviour in bumblebees; however, similar to the results obtained for honeybees, the situation appears complex, and it is not clear what could account for the decrease in feeding behaviour in these experiments. For instance, unexposed bumblebees that were provided with 100 μM or 200 μM nicotine (representing a dose of 10.1 and 15.1 μg/bee/day, respectively) dissolved in 0.5 M sucrose, reduced their feeding over the 24 h two-way choice period in contrast to their sucrose-only controls, however, bumblebees that were chronically pre-exposed to 100 μM or 200 μM nicotine in 0.5 M sucrose did not reduce the

total volume of solution that they consumed over the pre-exposure period, despite consuming a much higher dose of nicotine (20.0 and 32.7 μg/bee/day, respectively). This indicates that the dose of nicotine consumed was not responsible for the reduced feeding behaviour observed in unexposed bumblebees. i.e. if the dose of nicotine consumed was responsible for reducing feeding in the bumblebee, then bees that consumed a dose of 20.0 or 32.7 μg/bee/day would also be expected to decrease their consumption; however, this was not the case. Interestingly, bumblebees that were pre-exposed to 100 μM nicotine in 1.0 M sucrose (representing a dose of 11.3 μg/bee/day) did not exhibit a reduction in feeding in comparison to the sucrose-only control, however, bumblebees that were pre-exposed to 100 μM nicotine in 0.5 M sucrose (representing a dose of 20.0 μg/bee/day) did reduce their feeding. Although this may indicate that taste masking may be responsible for the decreased feeding observed in bees presented with nicotine in 0.5 M sucrose, this is unlikely, given that both unexposed and pre-exposed bumblebees did not find nicotine aversive in any of the 24 h two-choice tests. i.e. if bumblebees were decreasing their feeding because they found the taste of nicotine aversive, then aversion should have been present to identical concentrations of the compound in two-choice tests; however, this was not the case.

The results from the earlier experiments conducted in honeybees show that nicotine only suppressed feeding behaviour when honeybees were forced to chronically or intermittently consume nicotine in their only source of food over a number of days, whereas no suppressed feeding was observed in any of the unexposed treatments, even with nicotine concentrations as high as 500 μM. This is in contrast to the results obtained here, where both unexposed and pre-exposed bumblebees were shown to decrease the total volume that they consumed over the choice period when presented with 500 μM or 1000 μM nicotine in 0.5 M sucrose. This suggests that nicotine differentially suppresses feeding behaviour depending on the bee species and the schedule of administration used.

Previous studies conducted with imidacloprid, which is chemically similar to nicotine and also functions as a nAChR agonist (Palmer et al. 2013; Moffat et al. 2016), have identified that this compound can suppress feeding behaviour in bumblebees and honeybees (Kessler et al. 2015). Interestingly, similar to the results obtained here, imidacloprid has been shown to differentially affect the total volume of food consumed by

bees in a species-specific manner. Kessler et al. (2015) offered honeybees and buff-tailed bumblebees a 24 h two-way choice between imidacloprid presented in 0.5 M sucrose, or 0.5M sucrose alone. Bumblebees were shown to reduce the total volume of food they consumed over the 24 h choice period when as little as 1 nM imidacloprid was included in their food, in contrast, honeybees were not shown to reduce the total volume of food they consumed, even in response to imidacloprid concentrations as high as 1000 nM. This indicates that nAChR agonists differentially affect the feeding behaviour of different bee species. Furthermore, previous studies in the bumblebee subspecies *Bombus terrestris dalmatinus* have identified that nicotine concentrations as low as 1 μM can suppress feeding during a 24 h two-way choice test, using methods identical to those used in this thesis (Tiedeken et al. 2014). Again, this is in contrast to this study, where nicotine concentrations as high as 25 μM nicotine had no impact on the feeding behaviour of *Bombus terrestris audax* foragers in any of the experiments conducted. This indicates that not only does nicotine differentially affect the feeding behaviour of different bee species, but also bumblebee subspecies, although the reason for this is currently unclear.

4.4.4 Preferential nicotine consumption in the bumblebee: A comparison to previous studies

At present, only a small number of studies have examined whether bumblebees display a preference for nicotine in a two-way choice (Table 1.4). Tiedeken et al. (2014) identified that caged buff-tailed bumblebees (*B. terrestris*) were indifferent to low concentrations of the drug: 1 μM and 10 μM, and avoided nicotine at high concentrations: 100 μM and 1000 μM when administered in 0.5 M sucrose over a 24 h period. In a set of studies, Barrachi et al. (2015 & 2017a) identified that free-flying bumblebees (*B. terrestris*) foraging on artificial flowers in the laboratory exhibited a preference for flowers laced with 6 μM, but not 12 μM nicotine when delivered in 0.9 M sucrose, over flowers containing sucrose alone. Whereas bumblebees avoided flowers laced with higher concentrations of the drug (300 μM). However, bumblebees were shown to prefer 12 μM nicotine if they had previously been infected with the gut parasite *C. bombi* (Barrachi et al. 2015). Finally, Palmer-Young et al. (2017) identified no preference for 12 μM nicotine delivered in 0.9 M sucrose in the common eastern bumblebee (*B. impatiens*) when presented within a 24 h two-way choice in a caged setting within the laboratory. Collectively this indicates that

healthy, uninfected bumblebees exhibit a preference for low concentrations of nicotine (6 μM) in free-flight experiments, but are indifferent to marginally higher concentrations of the drug (10 μM and 12 μM), and avoid nicotine at higher concentrations (100-300 μM) in both a caged setting and in free-flight paradigms.

This study examined whether the buff-tailed bumblebee exhibited preferential nicotine consumption in a caged assay within the laboratory either during an immediate 24 h two-way choice (unexposed) or following 3 days or 5 days pre-exposure to the drug. Bumblebees were shown to display a significant preference for 100 nicotine μM only following 3 days and 5 days of pre-exposure in 1.0 M sucrose. Note that bumblebees in these experiments were purchased from Koppert Biological Systems, which conduct quality control to ensure colonies are disease-free (Huang et al. 2015). Therefore, although not explicitly tested, bumblebees in this experiment are assumed to be free from disease. In agreement with previous studies which have assessed preferential nicotine consumption in caged bumblebees, unexposed bees did not display a preference for low concentrations of the drug (6.25 – 25 μM). This is in contrast to free-flying bumblebees, which were shown to exhibit a preference for 6 μM nicotine in free-flight assays (Barrachi et al. 2017a).

In contrast to previous studies, aversion was not displayed for any nicotine concentration used, regardless of the concentration of sucrose the drug was administered in. Tiedeken et al. (2014) identified that buff-tailed bumblebees displayed an aversion to 100 μM nicotine when delivered in 0.5 M sucrose in a caged setting within the laboratory. Furthermore, Barrachi et al. (2017a) identified that free-flying bumblebees exhibited aversion for 300 μM nicotine when delivered in 0.9 M sucrose in free-flight assays. These discrepancies may be explained by the experimental paradigm used (free-flight versus caged bees) or the subspecies of bumblebee used.

Free-flight experiments are notably different to caged assays within the laboratory. For instance, in free-flight bumblebee assays, bees are required to make a set number of choices (e.g. 100 flowers visited), and preferences are identified by the number of visits to flowers containing the drug versus flowers that are absent for the drug. Due to the design of these experiments, bees are not given a set duration of drug access (e.g. 1 h); rather, they only need to reach their quota of flower visits. Barrachi et al. (2017a) used a criterion of

100 flower visits to assess preferential nicotine consumption. However, these authors did not provide the average foraging duration required to achieve this. Previous studies which have used similar free-flight paradigms have identified that bumblebees typically visit 8-10 artificial flowers/min (Heinrich et al. 1977; Otterstatter and Thompson, 2006; Mobley and Gegear, 2018). Therefore, a liberal estimate is that bumblebees in this assay were experiencing nicotine in only a very short time window, ~10-20 min.

This aversion to nicotine is therefore reminiscent of the aversion to nicotine observed in free-flight assays conducted in honeybees by Singaravelan et al. (2005). one possible explanation for the differences observed in short access free-flight experiments in comparison to caged assays may be the development of acute tolerance to the adverse effects of the nicotine brought on by prior experience with the drug, as occurs in rodent models (discussed in 3.4.3). If this is the case, then caged bumblebees allowed longer access to the drug may develop acute tolerance to the aversive effects of high doses of nicotine over time. This could explain why no aversion was observed for higher nicotine concentrations in my study. In agreement with the notion that free-flight paradigms may result in differential behaviour to that observed in a caged environment, Palmer-Young et al. (2017) identified that bumblebees did not display a preference for 12 μM nicotine over a 24 h choice period in a caged environment, whereas Barrachi et al. (2017a) identified a preference for the same concentration of nicotine in a free-flight assay.

Interestingly, Tiedeken et al. (2014) used an identical behavioural paradigm to the one used in this study; however, they identified that bumblebees avoided 100 and 1000 μM nicotine when delivered in 0.5 M sucrose. This discrepancy in avoidance behaviour may be explained by the subspecies of bee used. Tiedeken et al. (2014) used *B. terrestris dalmatinus*, in contrast to *B. terrestris audax* used in this study. Whereas *audax* is the native subspecies to the UK, *dalmatinus* is native to more southerly regions such as Italy, Iran, and Israel (Velthuisa and van Doornb, 2006; Rasmont et al. 2008).

Although *B. terrestris* subspecies are commonly discussed in the literature as a single homogenous group, it is important to note that geographically isolated subspecies of bumblebees differ in a wide number of aspects, such as their underlying genetics (Estoup et al. 1996; Moreira et al. 2015; Lecocq et al. 2013; Kent et al. 2018), rate of learning (Ings

et al. 2009), size (Ings et al. 2006), labial gland secretions (Coppée et al. 2008; Lecocq et al. 2013), and sensory perception such as visual spectral sensitivity (Skorupski et al. 2007; Raine and Chittka, 2007). The notion that subspecies may differ in their response to pharmacological agents has previously been confirmed in the honeybee, where subspecies differ in their sensitivity to neonicotinoids (Suchail et al. 2000), and in *Drosophila,* where tolerance to ethanol varies on the subspecies in question (Gao et al. 2018). In addition, as discussed above, the bumblebee subspecies used in this thesis did not display reduced feeding behaviour to concentrations of nicotine as high as 25 μM, whereas concentrations of just 1 μM were shown to suppress feeding in *B. terrestris dalmatinus*. Similar reports of strain-specific responses are well understood in the mammalian literature, where even a single nucleotide change in the α4-nAChR between rodent strains results in increased sensitivity to the aversive effects of nicotine (Portugal and Gould, 2008). Similar subspecies differences may therefore explain why bumblebees in this study did not avoid consuming intermediate concentrations of nicotine (100-500 μM).

4.4.5. Bumblebees display a preference for nicotine only following a period of forced pre-exposure

In agreement with previous bumblebee studies (Tiedeken et al. 2014; Palmer-Young et al. 2017), no preference was observed for nicotine in a 24 h two-way choice in a caged setting within the laboratory for any nicotine concentration tested. This is in agreement with the previous study in honeybees (chapter 3.0) and to what is observed in mammalian models, where rodents are largely indifferent to nicotine until they have experienced the drug for a period of ~7-14 days (Valentine et al. 1997; Fu et al. 2001; Brower et al. 2002; Fu et al. 2003; Parker et al. 2004).

Following both 3 and 5 days of pre-exposure to the drug bumblebees exhibited a significant preference for 100 μM nicotine in a 24 h two-way choice. This is again in agreement with rodent models of addiction which have identified that pre-exposing rodents to nicotine results in robust facilitation of nicotine self-administration when subsequently offered the opportunity to self-administer the drug (Natividad et al. 2013; Renda and Nashmi, 2014). Indeed, similar pre-exposure schedules have been used in *Drosophila* in order to generate preferential consumption of ethanol. For instance, Peru y Colón de

Portugal et al. (2014) identified that unexposed *Drosophila* find ethanol aversive; however, display a robust preference for the drug following a single day of ethanol pre-exposure.

The 5 day pre-exposure period was included as extended periods of nicotine administration in rodents are known to increase the level of dependency, as evidenced by increased symptoms and duration of withdrawal syndrome (Damaj et al. 2003; Skjei and Markou, 2003; Vann et al. 2006). Although smokers that exhibit higher levels of nicotine dependence are known to smoke more cigarettes (Bandiera et al. 2015; Mercincavage et al. 2018), to the best of my knowledge, length of pre-exposure on preferential nicotine administration in rodents has not yet been tested, therefore it is not clear if this human-like behaviour translates to animal models of addiction. Although increasing the duration in this study did not increase the level of nicotine preference, it may simply be that 5 days pre-exposure is an insufficient increase to result in increased nicotine preference in comparison to 3 days pre-exposure in the bumblebee.

Although bumblebees were shown to display a significant preference for solutions containing nicotine following a pre-exposure period, this study does not identify what serves as the primary motivator for this behaviour. Unlike the earlier study conducted in honeybees (chapter 3.0), where preferential consumption of nicotine was only observed following a period of intermittent access, suggestive of negative reinforcement behaviour, this study does not identify whether bumblebees preferentially choose nicotine due to positive reinforcement (i.e. presumed hedonic effects), or negative reinforcement (i.e. maintaining a sufficient dose of nicotine to avoid withdrawal). However, given that unexposed bumblebees did not display a preference for nicotine in a 24 h two-choice test suggests that nicotine does not function as a strong positive reinforcer in the bumblebee, suggesting that negative reinforcement brought on by pre-exposure is more likely. In order to confirm whether dependence (i.e. withdrawal) may be a contributing factor to the preferential consumption behaviour observed, direct assessment of nicotine withdrawal symptoms following 3 or 5 days nicotine pre-exposure in the bumblebee is required.

4.4.6. Pre-exposure to nicotine affects the chosen nicotine dose in the choice test

Similar to the results observed in adult forager honeybees (Chapter 3.0), in all cases during the 24 h two-way choice period, bumblebees selected a lower dose than they had experienced over the 3 or 5 day pre-exposure periods, indicating that bumblebees were not attempting to match the dose they were previously accustomed to, and is in agreement with previous studies conducted in mammals (discussed in detail 3.4.7).

4.4.7 Changing the concentration of sucrose the drug is administered in does not affect the preference for nicotine in the bumblebee

Previous studies have indicated that bees are less likely to reject alkaloids when presented in more concentrated sucrose solutions (Gegear et al. 2007; Köhler et al. 2012a), presumably, as an increased concentration of sucrose is more effective at 'masking' the bitter taste of these compounds (Cocco and Glendinning, 2012; Köhler et al. 2012a). Indeed, oral studies of nicotine in rodent models have similarly indicated that 'masking' the taste of nicotine in water through the addition of sucrose or saccharin can increase the total volume of nicotine voluntarily consumed by the rodent (Smith and Roberts, 1995; Robinson et al. 1996; Kasten et al. 2016).

In order to assess whether sucrose molarity affected the preference for nicotine in caged bumblebees during the 3 day pre-exposure period, experiments were repeated in both 0.5 M and 1.0 M sucrose. However, in agreement with the previous honeybee study (discussed in 3.4.3), increasing the sucrose concentration did not result in preferential consumption of nicotine over sucrose in any of the experiments conducted (Appendix: Supplementary Fig. 1.2). Therefore, although pre-exposed bumblebees were shown to preferentially consume 100 μM nicotine when delivered in 1.0 M sucrose, and no significant preference was observed when 100 μM nicotine was delivered in 0.5 M sucrose, the most parsimonious explanation for this is that this result arose due to the dose the bee consumed during the course of pre-exposure. For instance, bumblebees that were provided with 100 μM nicotine in 0.5 M sucrose consumed an average nicotine dose of 20 μg/bee/day over the pre-exposure period and chose a dose of 9.48 μg/bee/day over the choice period. In contrast, bumblebees that were provided with 100 μM nicotine in 1.0 M sucrose consumed an average nicotine dose of 11.3 μg/bee day over the pre-exposure

period and chose a dose of 7.02 μg/bee/day over the choice period. That is, bumblebees that were provided with nicotine in 0.5 M sucrose consumed approximately double the nicotine dose than bumblebees that were provided with nicotine delivered in 1.0 M sucrose over the pre-exposure period. Given that nicotine preferences arise in a biphasic dose-dependent manner in mammals (discussed in 1.7.2), this change in the dose consumed during the pre-exposure period may account for the change in preferential consumption. If this is the case, then this could be tested by adjusting the nicotine dose that bumblebees receive during the pre-exposure period when nicotine is delivered in 0.5 M sucrose, e.g. 50 μM nicotine in 0.5 M sucrose would provide approximately the same dose received in bumblebees that consumed 100 μM nicotine in 1.0 M sucrose.

4.4.8. Bumblebees do not prefer nicotine over an alternative reinforcer of greater magnitude; however, nicotine pre-exposure affects sucrose reward evaluation

Behavioural economics combines basic concepts from economics and behavioural psychology to assess the relative motivation to peruse rewarding stimuli (Correia et al. 2010). In addiction studies, behavioural economics is applied to assess the motivation to pursue a drug reward over an alternative reinforcer and can provide a measurement of the relative reinforcing properties of the drug (Correia et al. 2010). In human studies, this is typically assessed by offering a subject the choice between a drug or a monetary reward (Correia et al. 2010). Whereas, in rodents, this is assessed by offering the rodent a choice between an alternative drug (e.g. cocaine), or saccharin or sucrose sweetened water (Ahmed et al. 2010, Ahmed, 2018). How reinforcing the drug is to an animal can then be determined by altering the magnitude of the alternative reinforcer whilst maintaining the level of drug reinforcement (Correia et al. 2010; Ahmed et al. 2010, Ahmed, 2018). In terms of the DSM-V/ICD-10 criteria, preferences for alternative rewards over alternative reinforcers fall in line with a number of criteria. For instance, devaluation of natural rewards in comparison to drugs of abuse indicates both a strong desire or urge to continue drug use, as well as drug use at the cost of natural rewards, such as time spent with family or friends, or consumption of food, etc. This, in turn, arguably indicates continued use despite harm.

Only a single study to date has examined sub-optimal choice behaviour in response to nicotine in insects. Barrachi et al. (2017a) identified that even though bumblebees avoid high concentrations of nicotine (300 μM) in a two-choice test, they were more likely to remain faithful to flower colours that were associated with the nicotine-laced solution, in comparison to flower colours that contained 0.9 M sucrose alone, even when these flowers were made sub-optimal by replacing the nicotine-laced solution with water. This study indicates that nicotine likely interferes with cue-reward association learning in bumblebees and suggests that nicotine interferes with the bees reward circuitry, similar to mammals.

Bumblebees prefer high concentrations of sucrose (Konzmann and Lunau, 2014). Thus, if nicotine is capable of exerting addictive properties in the bee, it was expected that the pre-exposure period would decrease the bumblebees preference for higher molarity sucrose solutions during the 24 h choice test, i.e. higher molarity sucrose solutions would be devalued as the bumblebee would preferentially consume nicotine. Although the pre-exposed bumblebees did not exhibit an outright preference for the nicotine-containing solution when offered a choice between 1.1, 1.2, or 1.3 M sucrose, the significant interaction indicates that bumblebees are willing to partly forgo the higher molarity sucrose in order to continue consumption from the nicotine-containing solution. In contrast, previous studies have shown that bees that have never experienced nicotine reliably select higher molarity sucrose solutions when offered a choice between sucrose solutions of differing concentrations (Konzmann and Lunau, 2014). This result indicates that nicotine is exerting an effect on the bumblebees' evaluation of reward quality. Despite this effect, bumblebees were shown to display a significant preference for both 1.2 and 1.3 M sucrose regardless of their pre-exposure to nicotine.

There are currently only a handful of papers to date which have assessed whether rodents prefer nicotine over an alternative reinforcer (Manzardo et al. 2002; LeSage, 2009; Stairs et al. 2010; Kasten et al. 2016; Huynh et al. 2017; Russo et al. 2018). Rodents are known to prefer cocaine (Manzardo et al. 2002), ethanol (Kasten et al. 2016), and sucrose or saccharin (LeSage, 2009; Stairs et al. 2010; Huynh et al. 2017; Russo et al. 2018) over nicotine. It is important to note; however, that these studies used rodents that had only experienced nicotine in short access paradigms (1 h/day), a schedule of nicotine administration that does not result in nicotine dependence (Watkins et al. 1999; Baker et

al. 2013; Cohen et al. 2015). Therefore, non-dependent rodents do not perceive nicotine as more rewarding than alternative rewards of greater value.

Behavioural economics studies in dependent smokers have shown that the probability of choosing a cigarette over money decreases as the monetary value increases (Tidey et al. 1999; Johnson and Bickel, 2003; Bisaga et al. 2007; Stoops et al. 2011; McKee et al. 2012; Sweitzer et al. 2013; Businelle et al. 2014; Cassidy et al. 2015; Tidey et al. 2016; Motschman et al. 2018), and the propensity to choose nicotine over money depends on the severity of dependence in the individual (Sweitzer et al. 2013; Cassidy et al. 2015). Importantly, these studies have repeatedly shown that even heavily dependent smokers only exhibit a preference for cigarettes over money or other alternative reinforcers when they are in a deprived state following a period of nicotine abstinence (Epstein et al. 1991; Rusted et al. 1998; Tidey et al. 1999; Jenks and Higgs, 2011; Kollins et al. 2013). In other words, the true value of the drug to the individual only emerges when the drug is needed to alleviate withdrawal symptoms; provided the smoker is not currently experiencing withdrawal symptoms, the desire for nicotine is low, and the alternative reinforcer is instead preferred. This is in line with the notion that the primary addictive properties of nicotine are maintained by the compounds ability to facilitate negative reinforcement, as opposed to positive reinforcement (George and Koob, 2017). This suggests then, that the bumblebees devaluation of the higher molarity sucrose solution may be in part mediated by nicotine dependence, and the bees willingness to partly forgo the higher molarity sucrose may be due to the bees desire to avoid withdrawal syndrome.

Bumblebees in this study were offered a choice between nicotine or an alternative reinforcer immediately after they had completed a period of 3 days chronic pre-exposure, a time when haemolymph nicotine levels will be at their maximum (Du Rand et al. 2017). Therefore, if nicotine is functioning as an addictive agent in the bumblebee, they would not be in a withdrawn state when they entered the 24 h choice period due to high haemolymph levels of the alkaloid. However, withdrawal symptoms would be expected to slowly develop over the course of the 24 h period if the bees were to cease or substantially decrease their consumption from the nicotine-laced solution. If preferences for nicotine over alternative reinforcers of greater value are only obtained in subjects that are experiencing withdrawal, then it may be possible that if bumblebees were forced to

undergo a period of nicotine abstinence immediately prior to the choice day, that bumblebees would display a stronger devaluation of the higher molar sucrose solutions. That is, nicotine abstinence may increase the bumblebees motivation to consume nicotine during the choice period, in line with the increased motivation for nicotine observed in the honeybee following a period of forced nicotine abstinence (Chapter 3.0).

In line with the increased preference for 1.2 and 1.3 M sucrose solutions, the chosen nicotine dose was seen to decrease accordingly over the 24 h choice period. Bees were shown to consume a dose of 8.24 μg/bee/day when offered a choice between nicotine and 1.0 M sucrose, whereas they consumed 3.45, and 2.87 μg/bee/day when offered a choice for 1.2 and 1.3 M sucrose, respectively, representing a 2.9 fold decrease in dose consumed for the highest molarity sucrose. This indicates that bees not only preferred the two most nutritious sugars but that they were willing to decrease their dose of nicotine to preferentially consume from a higher molarity sucrose solution.

Although the decreased nicotine dose observed may be predicted to lead to withdrawal symptoms, and therefore negative reinforcement behaviour over the course of the 24 h choice period, it is important to note that the dose required to stave off withdrawal is reasonably flexible in both moderately dependent smokers and rodent models of nicotine addiction. Nicotine withdrawal is produced through a drop in nicotine concentration in the body, and the severity of withdrawal is a function of how dependent a subject is on the drug. For instance, human subjects only begin to enter a state of protracted withdrawal once their nicotine intake is gradually reduced from 12 mg to 4 mg over the course of 3 weeks, a 3-fold decrease (Benowitz et al. 2007). In addition, rodents have been shown to experience as much as a 35 % reduction in dose without experiencing symptoms of withdrawal (Harris et al. 2011). Therefore, bumblebees in this assay may be similarly flexible in their required dose, such that they can decrease their chosen dose to preferentially consume from the highest molarity sucrose solutions without risking entering a state of protracted withdrawal. If bumblebees require a specific dose of nicotine to avoid withdrawal, then decreasing the concentration of nicotine over the 24 h choice period (e.g. from 100 μM to 25 μM) may force the bee to compensate by increasing their overall consumption from the nicotine-containing solution. Such compensatory self-

administration behaviour is observed in rodents when nicotine concentrations are decreased sufficiently (Adriani et al. 2002a; Adriani et al. 2002b; Harris et al. 2011).

It is also important to note that alternative reinforcers themselves are capable of mitigating withdrawal due to their ability to activate reward circuitry (Helmers and Young, 1998; West et al. 1999; McRobbie and Hajek, 2004; Lui and Grigson, 2005; Berlin et al. 2005; Lussier et al. 2006; Segovia et al. 2010; Skwara et al. 2012). Nicotine abstinence attenuates dopamine release in the reward circuitry of rodents (Hildebrand et al. 1998; Carboni et al. 2000; Rada et al. 2001; Natividad et al. 2010; Zhang et al. 2012), due at least in part to increases in corticotropin releasing factor (CRF) (Grieder et al. 2014; Zhao-Shea et al. 2015, George and Koob, 2017), and negative reinforcement behaviour occurs due to a desire to rectify the hypo-dopaminergic state (George and Koob, 2017). Dopamine release in rodents increases as a function of sucrose concentration (Hajnal et al. 2004), and 0.3 M sucrose releases dopamine levels equivalent to that of low doses of nicotine (Brazell et al. 1991; Schilström et al. 1998; Rahman et al. 2003; Hajnal et al. 2004). Furthermore, sucrose consumption reduces stress through its action on CRF mRNA (Ulrich-Lai et al. 2007; Ulrich-Lai et al. 2010). Indeed, sucrose or glucose tablets have been shown to buffer nicotine withdrawal in human subjects (Helmers and Young, 1998; West et al. 1999; McRobbie and Hajek, 2004; Berlin et al. 2005), and increased intake of sweet, highly calorific foods, is a well-documented behavioural response to nicotine cessation in both humans (Rodin, 1987; Jo et al. 2002; Donny et al. 2011; Natividad et al. 2013; Komiyama et al. 2013; Bush et al. 2016), and rodents (Levin et al. 1987; Grunberg et al. 1985; Jias and Ellison, 1990; Bishop et al. 2002). Thus, the bee's act of consuming a higher molarity solution may in itself partly mitigate any effects of nicotine withdrawal that occur due to a reduced nicotine dose.

4.4.9. Mortality

Nicotine administration did not result in increased mortality relative to control in any of the experiments conducted. Previous experiments assessing mortality in bumblebees have only addressed low concentrations of the drug. For instance, no mortality was observed for 12 μM nicotine following 7 days chronic consumption (Richardson et al. 2015; Biller et al. 2015; Thorburn et al. 2015). In addition, Barrachi et al. (2015) fed

bumblebees 12 μM nicotine continually from their time of collection until their death and identified that bees chronically fed nicotine died on average only 4 days earlier than control bees fed sucrose alone (39 days vs 43 days). My work, in contrast, showed that chronic consumption of nicotine in doses from 3.37-104 μg/bee/day for a period of 3 or 5 days does not result in increased mortality. My subjects only consumed nicotine for a period of 4 days; therefore, extended chronic consumption of nicotine may have a different effect to shorter periods of chronic consumption, which may account for the differences observed between studies.

4.4.10. Conclusion

To the best of my knowledge, this serves as the only study to date that has identified that chronic pre-exposure to nicotine results in preferential consumption of the compound in any insect species. However, it is not currently clear in this instance what motivates the bees' behaviour, and further studies are required to ascertain whether positive reinforcement, negative reinforcement, or indeed both forms of reinforcement in synchrony are responsible for preferential nicotine consumption in the bumblebee. However, given that no preferences were observed for nicotine in unexposed bees, and preferences only emerged following pre-exposure, suggests that negative reinforcement is likely governing the behaviour observed.

The fact that bumblebees were shown to devalue higher molarity sucrose solutions indicates that nicotine pre-exposure likely interferes with the bees reward circuitry, however, this response alone is insufficient to deem bumblebees as exhibiting addictive-like behaviour in line with the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) or the World Health Organizations International Classification of Diseases (ICD10) criteria for addiction and further studies are required to ascertain if bumblebees meet sufficient criteria to be considered addicted to the alkaloid (e.g. evidence of withdrawal). Furthermore, in order to examine whether negative reinforcement plays a role in this devaluation behaviour, future studies may focus on decreasing the nicotine dose available to the bee over the choice period, including a period of forced nicotine abstinence prior to the choice period, and/or focusing on a narrower range of sucrose concentrations.

Chapter 5.0 Do honeybees and bumblebees prefer to consume caffeinated sucrose over sucrose alone?

5.1 Introduction

Caffeine is a purine alkaloid that is synthesised in a diverse number of plants (Ashihara, 2004). It is the world's most commonly used psychoactive drug (Grigg, 2002), and low doses in humans can increase wakefulness (Nehlig et al. 1992), attention (Pasman et al. 2017: Park et al. 2014), and mood (Ruxton, 2008), and may improve certain forms of memory (Nehlig, 1992). In spite of the fact that prolonged and repeated consumption of the drug is strongly associated with aspects of addiction in humans, such as tolerance and withdrawal (Meredith et al. 2013), evidence for true addiction is rare and still debated.

Although most well-known for its role as a central nervous system stimulant, caffeine, like nicotine, is believed to have evolved primarily as a natural pesticide to deter herbivores (Nathanson, 1984; Huang et al. 2016). Similar to nicotine, caffeine is both bitter and toxic to animals in high doses (Rosenthal and Berenbaum, 2012), and the concentrations of caffeine that are present in the leaves and seeds of the plant (Mazzafera, 1999; Mazzafera and Silvarolla, 2010) are known to deter herbivorous insects (Shields et al. 2008; Lee et al. 2009; Ignell et al. 2010; Asparch et al. 2016). Caffeine is found in low, non-toxic concentrations in floral nectar and pollen (Kretschmar and Baumann, 1999; Wright et al. 2013; Detzel and Wink, 1993), indicating a potential role for caffeine in mediating plant-pollinator interactions.

Laboratory-based assays of bumblebee foraging have shown that 'plants' that contain caffeinated food receive a greater rate of pollination over caffeine-free flowers (Thompson et al. 2015). These authors suggest that caffeine may serve to increase the fidelity of pollinators to the plants they pollinate, improving plant fitness. Additionally, free-flying honeybees have been shown to display a preference for caffeinated solutions over caffeine-free equivalents when presented in a two-way choice (Singaravelan et al. 2005). Caffeinated solutions were also shown to increase both the frequency and likelihood of honeybees performing the waggle dance (Couvillon et al. 2015), a behaviour that is known to correlate with the bees' valuation of nectar quality (Von Frisch, 1967; Seeley, 1991). Finally, it has recently been identified that honeybees trained to associate a floral

scent with a sucrose reward are three times more likely to remember that association three days later if the sucrose contained caffeine (Wright et al. 2013). Collectively this suggests that caffeine serves to reinforce bee behaviour; however, it is not clear whether caffeine is capable of functioning as an addictive agent in the bee, as it does in mammals.

Mammalian studies of caffeine addiction have established that in order to generate preferential consumption of caffeine in two-choice tests, the animals must first experience either forced chronic exposure (Vitiello and Woods, 1975; Vitiello and Woods, 1977; Griffiths et al. 1986; Newland and Brown, 1992; Fedorchack et al. 2002) or intermittent periods of pre-exposure (Myers and Izbicki, 2006), prior to the test. Immediate (unexposed) preferences for caffeine in both rodents and humans are rarely observed and are only present in extremely low concentrations (Heppner et al. 1986; Tordoff et al. 2008; Vautrin et al. 2005; Evans and Griffiths, 1992). Interestingly, previous studies identified a preference for caffeinated food in honeybees (Singaravelan et al. 2005; Couvillon et al. 2015), but these studies used brief (1-3 h) caffeine exposure periods, and no studies have yet assessed whether, similar to mammals, long-term exposure affects a preference for caffeine in the bee. Given that honeybees and bumblebees were previously shown to display a preference for nicotine, another plant alkaloid, following intermittent or chronic schedules of administration, here, it was tested whether forced pre-exposure, or intermittent feeding schedules affect an observed preference for caffeinated food in groups of adult worker honeybees and individual worker bumblebees.

5.2 Methods

Honeybees were exposed to 6 different feeding schedules in these experiments: (i) unexposed (ii) 3 day pre-exposure, (iii) intermittent 12 (I12), (iv) intermittent 48 (I48), and (v) extended 48 h intermittent (EXT48) (see General methods). Bumblebees were exposed to 2 different feeding schedules in these experiments: (i) unexposed and (ii) 3 day pre-exposure (see general methods). The EXT48 exposure schedule was included in the honeybee experiments as honeybees were shown to prefer nicotine following the I48 schedule of administration (see chapter 3.0). Given that the number of cycles of drug administration and abstinence is believed to be important in generating a dependent state

in animals (Gilpin et al. 2014), this extended protocol was included to see if the addition of an extra abstinence cycle influenced the honeybees response to caffeine.

The concentrations of sucrose and caffeine used in these experiments are detailed in table 5.2. Caffeine concentrations for the unexposed and 3 day pre-exposure experiments were selected as they encompass the concentrations known to be both preferred and avoided in prior experiments in honeybees (Singaravelan et al. 2005; Liao al. 2017) and are within the range of caffeine concentrations found in floral nectar 3 – 1100 μM (Kretschmar and Baumann, 1999; Wright et al. 2013; Prado et al. 2019). For the honeybee intermittent treatments, 25 μM and 100 μM caffeine was used. These concentrations were selected as they would allow for a comparison to the intermittent schedules used for nicotine in earlier experiments.

No studies have assessed preferential consumption of caffeine in bumblebees; however, concentrations of caffeine ≤ 1000 μM are known to be consumed by bumblebees without aversion (Tiedeken et al. 2014). For this reason, a broader range of caffeine concentrations was used in the initial bumblebee experiments; unexposed and pre-exposed to caffeine delivered in 0.5 M sucrose (table 5.2). Given the results of these initial bumblebee experiments, it was clear that bumblebees were largely indifferent to high concentrations of the compound (≥ 500 μM). Therefore, for the unexposed and pre-exposed treatments in 1.0 M sucrose, a narrower range of caffeine concentrations was used (≤ 100 μM).

The total sugar concentration found in the nectar of plants that produce caffeine is typically very low (0.338 M – 0.843 mM) (Wright et al. 2013; Prado et al. 2019). Maintaining cohorts of honeybees or individual bumblebees in cages for prolonged periods on such low sugar concentrations is difficult due to the low carbohydrate content of the food. Previous literature assessing honeybee and bumblebee responses to caffeine have used sucrose concentrations between 0.5 - 1.0 M (Singaravelan et al. 2005; Mustard et al. 2012; Tiedeken et al. 2014; Wright et al. 2013). In keeping with the literature, and allowing a practical means of feeding bees for prolonged periods, sucrose concentrations of 0.5 and 1.0 M were used in these experiments (detailed in table 5.2). As honeybee and bumblebees aversion for food containing bitter substances, such as alkaloids (Gegear et al. 2007; Köhler

et al. 2012a) and phenolics (Lui et al. 2007), can be offset by increasing the concentration of sucrose that the compound is administered in, both 0.5 M and 1.0 M sucrose were tested in the bumblebee experiments. Unfortunately, due to the timing of experiments, it was not possible to include both sucrose concentrations in honeybees; therefore, 1.0 M sucrose was selected as 1.0 M sucrose would have a greater ability to mask the bitter taste of caffeine. Note that in all cases, the unexposed experiments were run separately from the pre-exposed experiments; however, the experiments were conducted at the same time of year to account for seasonal variability. e.g. for the 1.0 M sucrose experiments conducted in the honeybee, the unexposed treatments were conducted 1-3 weeks prior to the 3 day pre-exposure treatments.

Table 5.2 | Caffeine concentrations (µM) and sucrose concentrations (M) used in the bumblebee and honeybee choice experiments. Intermittent 12 (I12), intermittent 48 (I48), extended intermittent 48 (EXT48).

	B. terrestris	***B. terrestris***	***A. mellifera***
Feeding Schedule	**0.5 M sucrose**	**1.0 M sucrose**	**1.0 M sucrose**
Unexposed	0, 10, 100, 500, 1000	0, 10, 25, 100	0, 5, 10, 100
Pre-exposure: 3 day	0, 10, 100, 500, 1000	0, 10, 25, 100	0, 5, 10, 100
I12/I48/EXT48	N/A	N/A	0, 25, 100

5.3 Results

5.3.1 The response of unexposed and pre-exposed honeybees to different concentrations of caffeine in 0.5 M sucrose

Honeybees varied their total consumption across the pre-exposure period (Fig. 5.3.1a. RM-GLM, time, $F_{(2.48,253)} = 73.4$, $\eta_p^2 = 0.418$, $p = < 0.001$). They consumed as much as 85 µl of solution on the first day; on the second day, they consumed approximately 30 µl less food, and on the third day they consumed a similar volume to the first day. The inclusion of caffeine within the solution did not cause honeybees to consume more solution than the control (Extended data table 5.3.1.1a).

The data for the 24 h choice test show that exposure to caffeine in food for prolonged periods influenced subsequent choice behaviour (Fig 5.3.1b). Honeybees exposed to 5 µM caffeine in sucrose for 3 days were more likely to exhibit a preference for solutions containing 5 µM caffeine (Fig 5.3.1b. Extended data table 5.3.1.1b. two-way GLM, concentration x treatment, $F_{(3,230)} = 6.05$, $\eta_p^2 = 0.073$, $p = < 0.001$. *Post hoc* LSD, $p = <0.001$). In contrast, unexposed honeybees found 5 µM caffeine aversive, i.e. they preferred the sucrose solution to the caffeinated solution (Fig 5.3.1b. Extended data table 5.3.1.1b. *post hoc* LSD, $p = <0.001$). However, the opposite was true of honeybees pre-exposed to 10 µM caffeine; these bees avoided caffeine if exposed for 3 days, but unexposed honeybees preferred it (Fig 5.3.1b. Extended data table 5.3.1.1b. Note, *p*-value is marginal, *post hoc* LSD, $p = 0.087$). Neither the control nor the honeybees exposed to 100 µM caffeine displayed a preference or aversion (Fig. 5.3.1b. Extended data table 5.3.1.1b).

Honeybees in the pre-exposed treatment group consumed a significantly lower total volume of solution during the 24 h choice period than the unexposed treatment group for all caffeine concentrations (Fig. 5.3.1c Extended data table 5.3.1.1c. GLM with treatment set as main effect, $F_{(1,225)} = 68.4$, $\eta_p^2 = 0.233$, $p = <0.001$). However, note that on average, caffeine-exposed bees did not consume less than their specific sucrose-only control group (Fig. 5.3.1a & 5.3.1c) (Extended data table 5.3.1.1c).

Honeybees: Three day pre-exposure to caffeine in 1.0 M sucrose

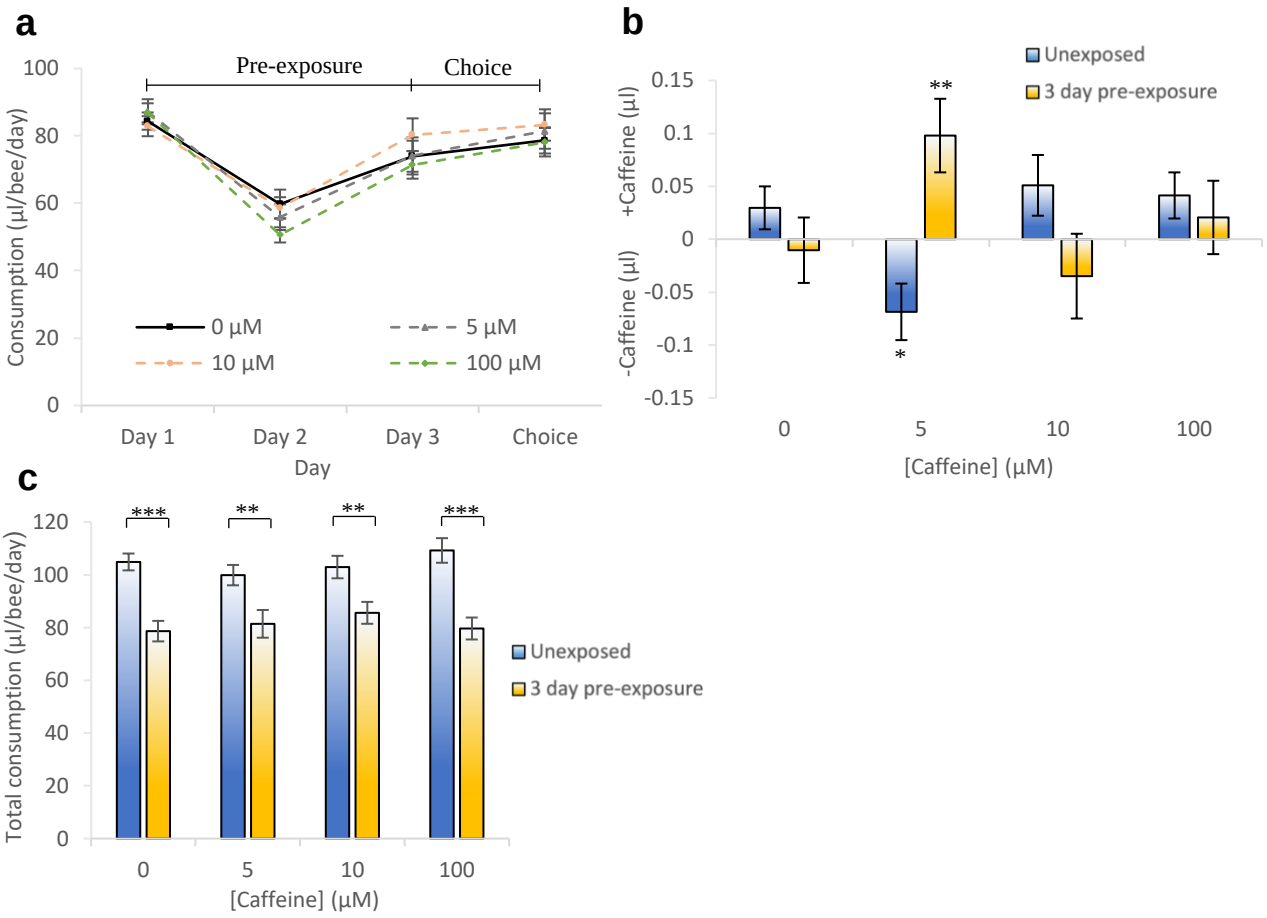

Figure 5.3.1 | Unexposed honeybees displayed an aversion for 5 µM caffeine, whereas they preferred 5 µM caffeine following 3 days pre-exposure. (a) Pre-exposure to caffeine did not affect the total volume consumed across the 3 day pre-exposure or 24 h two-way choice day periods in comparison to control (Extended data table 5.3.1.1a). **(b)** Unexposed honeybees displayed an aversion for 5 µM caffeine, whereas pre-exposure to caffeine for 3 days resulted in a preference for the caffeinated solution (Extended data table 5.3.1.1b). Conversely, unexposed honeybees displayed a weak preference for 10 µM caffeine compared to 3 day pre-exposed honeybees (Extended data table 5.3.1.1b). Data represent the mean difference in the amount consumed over 24 h; positive values indicate a preference for caffeine and negative avoidance of caffeine. Asterisks indicate significant differences for one-sample T-tests against 0 (*p <0.05; **p <0.01 **(c)** Honeybees in the pre-exposed treatment group consumed a significantly lower total volume of solution during the 24 h choice period than the unexposed treatment group for all caffeine concentrations (Extended data table 5.3.1.1c). However, on average, caffeine-exposed bees did not consume less than their specific control group (Extended data table 4.1c). Asterisks indicate significant differences for *post hoc* comparisons between treatments (LSD. **p <0.01; *** p <0.001). Bars and line graphs indicate mean (±s.e.m.) of consumption (µl) per bee, controlled for by evaporation.

The total dose that the bees consumed over the course of the experiment was also measured (Dose table 5.3.1). The dose of caffeine among the pre-exposed bees was as much 2-19x greater than the lowest caffeine concentration, depending on the treatment (Welch's ANOVA, $F_{(2,76)} = 999$, $\eta_p^2 = 0.964$, $p = <0.001$. Dose table 5.3.1a). For example, honeybees provided with 100 μM caffeine consumed a dose of 1350 ng/bee/day, whereas those provided with 5 μM solutions consumed an average dose of 70 ng/bee/day over the course of pre-exposure. During the 24 h choice test, the pre-exposed bees consumed a significantly lower dose of caffeine than the unexposed honeybees (GzLM, concentration x treatment, $\chi^2_{(2)} = 9.30$, $p = 0.009$. Extended data table 5.3.1.2c), with the exception of the 5 μM pre-exposed bees, which consumed similar a dose to the 5 μM unexposed bees (*Post hoc* LSD, $p = 0.654$). In all cases, the bees consumed less caffeine than they were exposed to during the 3 day pre-exposure period (Dose table 5.3.1, Extended data table 5.3.1.2e). For example, bees pre-exposed to 5 μM caffeine consumed 70.2 ng/bee/day during the pre-exposure period, but during the test, they chose a dose of 43.6 ng/bee/day.

Total mortality was not significantly different between control and treatment groups in unexposed honeybees (Kruskal Wallis test of differences, $\chi^2_{(3)} = 5.931$, $p = 0.115$). In the pre-exposed treatments, honeybees provided with 100 μM caffeine were more likely to survive till the end of the experiment than all other treatments (Kruskal Wallis test of differences, $\chi^2_{(3)} = 8.939$, $p = 0.03$. *Post hoc* two-tailed Mann-Whitney U tests against control for 100 μM caffeine, $U = 276.50$, $p = 0.009$, $\eta^2 = 0.114$).

Extended data table 5.3.1.1 | *A. mellifera* statistics for 24 h and 3 day pre-exposure to caffeine. Data correspond to figure 5.3.1a-c. **(a)** RM-GLM for the total consumption across the 3 day pre-exposure and 24 h two-way choice period. n = 0 μM (30), 5 μM (26), 10 μM (26), 100 μM (25). **(b)** GLM 24 h two-way choice data for unexposed and pre-exposed honeybees. n for unexposed 24 h data: 0 μM (29), 5 μM (29), 10 μM (28), 100 μM (29). n for pre-exposure choice data: 0 μM (30), 5 μM (26), 10 μM (29), 100 μM (28). **(c)** GLM for the total consumption during the 24 h two-way choice period for unexposed and pre-exposed honeybees and One-Way ANOVAs for each treatment.

(a)†	Time: $F_{(2.48, 253)} = 73.4$, $\eta p2 = 0.418$, $p = <0.001$ Concentration: $F_{(3, 102)}$, $= 0.241$, $\eta_p^2 = 0.007$, $p = 0.868$ Time x Concentration: $F_{(7.45, 253)} = 1.029$, $\eta_p^2 = 0.029$, $p = 0.413$ *Post hoc* pairwise comparisons for time (LSD): Day 1 vs day 2 $p = <0.001$, day 1 vs day 3 $p = < 0.001$, day 1 vs choice day $p = 0.058$, day 2 vs day 3 $p = <0.001$, day 2 vs choice day $p = <0.001$, day 3 vs choice day $p = 0.003$.
(b)	Treatment: $F_{(1, 230)} = 0.052$, $\eta_p^2 = <0.001$, $p = 0.820$ Concentration: $F_{(3, 230)} = 0.245$, $\eta_p^2 = 0.003$, $p = 0.865$ Treatment x Concentration: $F_{(3, 230)} = 6.053$, $\eta_p^2 = 0.073$, $p = <0.001$ *Post hoc* pairwise comparisons between treatments at each concentration (LSD): 0 μM $p = 0.363$, 5 μM $p = <0.001$, 10 μM $p = 0.056$, 100 μM $p = 0.617$ One sample T-tests against zero (LSD): unexposed 24 h two-way choice 0 μM $t_{(28)} = 1.46$ $p = 0.156$, 5 μM $t_{(28)} = -2.57$ $p = 0.016$, 10 μM $t_{(27)} = 1.77$ $p = 0.087$, 100 μM $t_{(28)} = 1.90$ $p = 0.068$. 3 day 24 h two-way choice 0 μM $t_{(29)} = -0.336$ $p = 0.739$, 5 μM $t_{(25)} = 02.82$ $p = 0.009$, 10 μM $t_{(28)} = -0.871$ $p = 0.391$, 100 μM $t_{(27)} = 0.151$ $p = 0.881$.
(c)	Treatment: $F_{(1, 225)} = 68.4$, $\eta p2 = 0.223$, $p = <0.001$ Concentration: $F_{(3, 225)} = 0.125$, $\eta_p^2 = 0.002$, $p = 0.945$ Treatment x Concentration: $F_{(3, 225)} = 0.939$, $\eta_p^2 = 0.012$, $p = 0.423$ *Post hoc* pairwise comparisons between treatments at each concentration (LSD): 0 μM $p = <0.001$, 5 μM $p = 0.003$, 10 μM $p = 0.001$, 100 μM $p = <0.001$ One-Way ANOVA for unexposed bees: $F_{(4, 130)} = 0.805$ $\eta_p^2 = 0.024$ $p = 0.524$ One-Way ANOVA for pre-exposed bees: $F_{(3, 102)} = 0.213$ $\eta_p^2 = 0.006$ $p = 0.887$

† Mauchly's test indicated that the assumption of sphericity has been violated ($\chi^2_{(5)} = 40.4$, $p = <0.001$), therefore Huynh-Feldt corrected tests are reported ($\varepsilon = 0.827$). n = 0 μM (30), 5 μM (26), 10 μM (26), 100 μM (25).

Dose table 5.3.1 | Summary of the average dose consumed by *A. mellifera* following consumption of caffeine in the 24 h two-way choice periods and during the pre-exposure period. Data correspond to figure 5.3.1a-c. Values represent the average caffeine dose consumed per individual honeybee (ng/bee/day). Green indicates the dose where preference is observed and red where avoidance is observed.

[Caffeine] µM	Pre-exposure period dose	Chosen dose	
		Pre-exposed	Unexposed
	ng/bee/day	ng/bee/day	ng/bee/day
5	70.2	**43.6**	**45.2**
10	144	75.7	104
100	1350	755	1100

Extended data table 5.3.1.2 | *A. mellifera* dosage statistics for 24 h and 3 day pre-exposure to caffeine. Data correspond to figure 5.3.1a-c and dose table 5.3.1. Welch's ANOVA for **(a)** the average dose consumed across the 3 day pre-exposure period **(b)** the unexposed bees 24 h two-way choice data, and **(c)** the 3 day pre-exposed 24 h two-way choice periods. *Post hoc* values are all possible pairwise comparisons between each concentration, Games-Howell adjusted. **(d)** GzLM for the chosen dose during the 24 h two-way choice between unexposed and pre-exposed bees. **(e)** GzLM for average dose consumed during the pre-exposure period and the dose chosen by pre-exposed bees during the choice test

(a) $F_{(2, 76)} = 999$ $\eta_p^2 = 0.964$ $p = <0.001$. All *post hoc* value comparisons $p = <0.001$.

(b) $F_{(2, 44.3)} = 343$ $\eta_p^2 = 0.923$ $p = <0.001$. All *post hoc* value comparisons $p = <0.001$.

(c) $F_{(2, 45.8)} = 172$ $\eta_p^2 = 0.807$ $p = <0.001$. All *post hoc* value comparisons $p = <0.001$.

(d) Concentration: $\chi^2_{(2)} = 2950$ $p = <0.001$

Treatment: $\chi^2_{(1)} = 25.7$ $p = <0.001$

Concentration x treatment: $\chi^2_{(2)} = 9.30$ $p = 0.009$

Post hoc comparisons between treatments at each concentration (LSD):

5 µM $p = 0.654$, 10 µM $p = <0.001$, 100 µM $p = <0.001$.

(e) Concentration: $\chi^2_{(2)} = 2950$ $p = <0.001$

Treatment: $\chi^2_{(1)} = 135$ $p = <0.001$

Concentration x treatment: $\chi^2_{(2)} = 1.90$ $p = 0.388$

Post hoc comparisons between treatments at each concentration (LSD):

5 µM $p = <0.001$, 10 µM $p = <0.001$, 100 µM $p = <0.001$.

5.3.2. The response of honeybees to 25 µM and 100 µM caffeine following an intermittent pre-exposure schedule in 1.0 M sucrose

Mean total consumption of solution at each period over the experiment was a function of the schedule of exposure (Extended data table 5.3.2a). Honeybees pre-exposed to caffeine in the I12 treatment showed a distinct pattern of total food consumption that varied as a function of time and caffeine concentration (Fig 5.3.2a. RM-GLM, time x concentration: $F_{(6.73, 172)} = 2.97$, $\eta_p^2 = 0.104$, $p = 0.006$). The honeybees in the I48 treatment showed a distinct pattern of consumption over time (Fig 5.3.2b. RM-GLM, time, $F_{(1.67,35.4)} = 72.9$, $\eta_p^2 = 0.776$, $p = <0.001$) that did not vary as a function of caffeine concentration (RM-GLM, time x concentration, $F_{(3.34,35.4)} = 1.18$, $\eta_p^2 = 0.101$, $p = 0.333$). The honeybees in the EXT48 treatment varied their total consumption as a function of time and caffeine concentration (Fig. 5.3.2c. RM-GLM time x concentration: $F_{(5.82,75.8)} = 3.41$, $\eta_p^2 = 0.202$, $p = 0.006$). Honeybees consumed as much as 84 µl over the initial 12 h period. This decreased to ~37 µl during the following 12 h. Total food consumption then oscillated over the remaining 2 days for all treatment groups.

None of the intermittent schedules produced a significant preference for caffeinated solutions during the 24 h two-choice test assay (Fig 5.3.2d: GLM treatment x concentration, $F_{(4,104)} = 0.756$, $\eta_p^2 = 0.028$, $p = 0.556$. Extended data table 5.3.2d). A marginal preference was observed for the 100 µM treatment in the EXT48 schedule (One sample T-test against 0, $T= 1.857_{(9)}$, $d = 0.59$, $p = 0.09$. Extended data table 5.3.2a), however, note that the sample size in this experiment was n = 10 treatment, suggesting that these experiments may lack enough statistical power to identify a difference.

Honeybees were shown to vary their total consumption over the 24 h choice period as a function of treatment (Fig. 5.3.2.e. GLM, main effect of treatment: $F_{(2, 99)} = 14.2$, $\eta p2 = 0.224$, $p = <0.001$. Extended data table 5.3.2.e). Honeybees in the I48 schedule consumed a significantly lower total volume of solution than bees that received the I12 or EXT48 schedules of administration (*post hoc* LSD, $p = <0.001$ and $p = <0.001$, respectively). Analysis of the honeybees total consumption within treatments identified that honeybees in the EXT48 group consumed significantly more food over the 24 h two-way choice period than bees that consumed sucrose alone (*post hoc* Bonferroni, p = 0.062 and 0.019. Note marginal *p*-value. Extended data table 5.3.2.e).

Honeybees: Intermittent pre-exposure to caffeine in 1.0 M sucrose

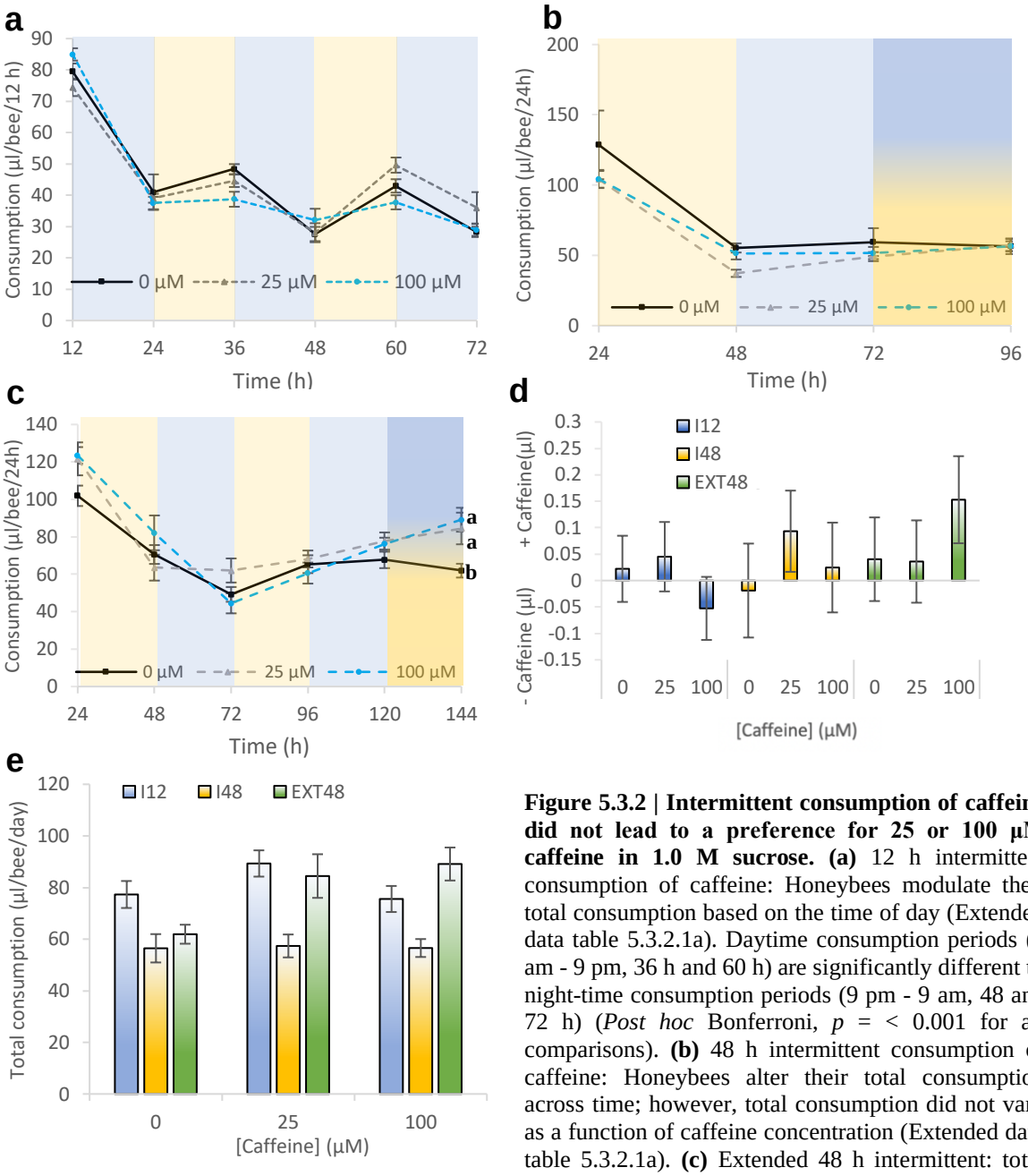

Figure 5.3.2 | Intermittent consumption of caffeine did not lead to a preference for 25 or 100 µM caffeine in 1.0 M sucrose. (a) 12 h intermittent consumption of caffeine: Honeybees modulate their total consumption based on the time of day (Extended data table 5.3.2.1a). Daytime consumption periods (9 am - 9 pm, 36 h and 60 h) are significantly different to night-time consumption periods (9 pm - 9 am, 48 and 72 h) (*Post hoc* Bonferroni, p = < 0.001 for all comparisons). **(b)** 48 h intermittent consumption of caffeine: Honeybees alter their total consumption across time; however, total consumption did not vary as a function of caffeine concentration (Extended data table 5.3.2.1a). **(c)** Extended 48 h intermittent: total consumption depends on the schedule and the concentration of caffeine in food (Extended data table 5.3.2.1b). Honeybees provided with 25 µM and 100 µM caffeine on the EXT48 schedule consumed a greater total volume on the choice day in comparison to the control (0 µM). Different letters indicate statistically significant differences between groups (*Post hoc* Bonferroni, p = 0.048 and 0.011, respectively). Yellow regions indicate periods of caffeine exposure and blue periods of sucrose exposure. **(d)** Honeybees that had received the I12, I48, or EXT48 pre-exposure schedules did not display a preference for 25 or 100 µM caffeine in a 24 h two-way choice (Extended data table 5.3.1.1d). Data represent the mean difference in the amount consumed over 24 h; positive values indicate a preference for caffeine and negative avoidance of caffeine. Bars and line graphs indicate mean (±s.e.m.) of consumption (µl) per bee controlled for by evaporation. **(e)** Honeybees that received the I48 schedule of pre-exposure consumed a lower total volume of solution over the 24 h two-way choice period than bees that received the I12 or EXT48 schedules of pre-exposure (extended data table 5.3.2.e).

Total mortality was not significantly different between control and treatment groups in the I12 (Kruskal Wallis test of differences, $\chi^2_{(2)} = 2.11$, $p = 0.348$), I48 (Kruskal Wallis test of differences, $\chi^2_{(2)} = 2.57$, $p = 0.276$), or EXT48 (Kruskal Wallis test of differences, $\chi^2_{(2)} = 0.422$, $p = 0.810$) feeding schedules.

Extended data table 5.3.2 | *A. mellifera* statistics for intermittent schedule 24 h two-way choice and total consumption. Data correspond to figure 5.3.2.a-c. RM-GLM for the total consumption across time for **(a)** I12: n = 0 μM (18), 25 μM (18), 100 μM (18). **(b)** I48: n = 0 μM (4), 25 μM (10), 100 μM (10), and **(c)** EXT48: n = 0 μM (10), 25 μM (10), 100 μM (10). **(d)** GLM for the indexed consumption for unexposed bees and pre-exposed bees during the 24 h two-way choice period. n I12: 0 μM (19), 25 μM (19), 100 μM (19); I48 0 μM (5), 25 μM (10), 100 μM (10); EXT48 0 μM (10), 25 μM (11), 100 μM (10). (e) GLM for the total volume consumed over the 24 h two-way choice period for unexposed and pre-exposed bees and One-Way ANOVAs for each treatment.

(a)†	Time: $F_{(3.37, 172)} = 138$, $\eta_p^2 = 0.731$, $p = <0.001$ Concentration: $F_{(2,51)} = 1.25$, $\eta_p^2 = 0.047$, $p = 0.294$ Time x Concentration: $F_{(6.73, 172)} = 2.97$, $\eta_p^2 = 0.104$, $p = 0.006$ *Post hoc* pairwise comparisons for time (Bonferroni): 12 h vs 24 h $p = <0.001$, 12 h vs 36 h $p = <0.001$, 12 h vs 48 h $p = <0.001$, 12 h vs 60 h $p = <0.001$, 12 h vs 72 h $p = <0.001$, 24 h vs 48 h $p = <0.001$, 30 h vs 48 h $p = <0.001$, 30 h vs 72 h $p = <0.001$ 48 h vs 60 h $p = <0.001$, 60 h vs 72 h $p = <0.001$. All other pairwise comparisons p = >0.05. *Post hoc* pairwise comparisons time x concentration (Bonferroni): 25 μM 12 h vs 12 h 100 μM $p = 0.012$, 36 h 0 μM vs 36 h 100 μM $p = 0.004$, 25 μM 60 h vs 100 μM 60 h $p = 0.002$. All other pairwise comparisons $p = >0.05$.
(b) ††	Time: $F_{(1.67, 35.4)} = 72.9$, $\eta p2 = 0.776$, $p = <0.001$ Concentration: $F_{(2, 21)} = 2.60$, $\eta_p^2 = 0.198$, $p = 0.098$ Time x Concentration: $F_{(3.34, 35.4)} = 1.18$, $\eta_p^2 = 0.101$, $p = 0.333$ *Post hoc* pairwise comparisons for time (Bonferroni): 24 h vs 48 h $p = <0.001$, 24 h vs 72 h $p = <0.001$, 24 h vs 96 h $p = <0.001$, 24 h vs 96 h $p = 0.040$. All other pairwise comparisons $p = >0.05$.
(c) †††	Time: $F_{(2.81, 75.8)} = 54.7$, $\eta p2 = 0.669$, $p = <0.001$ Concentration: $F_{(2, 27)} = 1.79$, $\eta_p^2 = 0.117$, $p = 0.186$ Time x Concentration: $F_{(5.82, 75.8)} = 3.41$, $\eta_p^2 = 0.202$, $p = 0.006$ *Post hoc* pairwise comparisons for time (Bonferroni): 24 h vs 48 h $p = <0.001$, 24 h vs 72 h $p = <0.001$, 24 h vs 96 h $p = <0.001$, 24 h vs 96 h $p = 0.040$, 24 h vs 120, $p = <0.001$, 24 h vs 144 h $p = <0.001$, 48 h vs 60 h $p = 0.016$, 60 h vs 72 h $p = <0.001$, 70 h vs 96 h $p = <0.001$, 70 h vs 120 h $p = <0.001$, 72 h vs120 h $p = <0.001$. All other pairwise comparisons $p = >0.05$.

	Post hoc pairwise comparisons time x concentration (Bonferroni): 0 µM 144 h vs 25 µM 144 h p = 0.048, 0 µM 144 h vs 100 µM 144 h p = 0.011. All other pairwise comparisons p = >0.05.
(d)	Treatment: $F_{(2, 104)}$ = 0.741, η_p^2 = 0.014, p = 0.479 Concentration: $F_{(2, 104)}$ = 0.207, η_p^2 = 0.004, p = 0.813 Treatment x Concentration: $F_{(4, 104)}$ = 0.756, η_p^2 = 0.028, p = 0.556 One sample T-tests against 0 (LSD), I12: 0 µM $t_{(18)}$ = 0.453 p = 0.727 , 25 µM $t_{(18)}$ = 0.687 p = 0.501, 100 µM $t_{(18)}$ = -0.883 p = 0.389. I48: 0 µM $t_{(4)}$ = 2.13 p = 0.842, 25 µM $t_{(9)}$ = 1.21 p = 0.255, 100 µM $t_{(9)}$ = 0.290 p = 0.778, EXT: 0 µM $t_{(9)}$ = 0.509 p = 0.623, 25 µM $t_{(10)}$ = 0.462 p = 0.654, 100 µM $t_{(9)}$ = 1.86 p = 0.096.
(e) ††††	Treatment: $F_{(2, 99)}$ = 14.2, $\eta p2$ = 0.224, p = <0.001 Concentration: $F_{(2, 99)}$ = 2.65, η_p^2 = 0.051, p = 0.076 Time x Concentration: $F_{(4, 99)}$ = 2.28, η_p^2 = 0.084, p = 0.066 *Post hoc* pairwise comparisons between treatments (LSD): I12 vs I48 p = <0.001, I48 vs EXT48 p = <0.001. All other pairwise comparisons p = >0.05. One-Way ANOVA for I12 bees: $F_{(2, 51)}$ = 2.14 η_p^2 = 0.077 p = 0.128 One-Way ANOVA for I48 bees: $F_{(2, 21)}$ = 0.013 η_p^2 = 0.001 p = 0.987 One-Way ANOVA for EXT48 bees: $F_{(2, 27)}$ = 5.04 η_p^2 = 0.272 p = 0.014 Post hoc (Bonferroni): 0 µM vs 25 µM p = 0.062, 0 µM vs 100 µM p = 0.019, 25 µM vs 100 µM p = 1.000.

† Mauchly's test indicated that the assumption of sphericity has been violated ($\chi^2_{(14)}$ = 70.7, p = <0.001), therefore Greenhouse-Geisser corrected tests are reported (ε = 0.673).

†† Mauchly's test indicated that the assumption of sphericity has been violated ($\chi^2_{(5)}$ =26.7, p = <0.001), therefore Greenhouse-Geisser corrected tests are reported (ε = 0.556).

††† Mauchly's test indicated that the assumption of sphericity has been violated ($\chi^2_{(14)}$ =37.0, p = <0.001), therefore Greenhouse-Geisser corrected tests are reported (ε = 0.562).Data was square-root transformed.

††††Data was log10 transformed.

5.3.3 The response of unexposed and pre-exposed bumblebees to different concentrations of caffeine administered in 0.5 M sucrose

Bumblebees pre-exposed to caffeine did not consume significantly more solution than the control over the 3 day pre-exposure period (Fig. 5.3.3a RM-GLM, time x concentration, $F_{(12,291)} = 0.792$, $\eta_p^2 = 0.032$, $p = 0.659$) (Extended data table 4.4a).

During the 24 h choice test, bumblebees did not exhibit a preference for caffeinated solutions (Fig. 5.3.3b GLM, concentration x treatment, $F_{(4,202)} = 0.238$, $\eta_p^2 = 0.005$, $p = 0.916$. Extended data table 5.3.3.1b). Although both unexposed and pre-exposed bumblebees showed a weak preference for 10 μM caffeine, this result was not significant (Extended data table 5.3.3.1b). None of the higher concentrations of caffeine elicited either preference or aversion.

Bumblebees in the pre-exposed treatment group consumed a significantly lower total volume of solution during the 24 h choice period than the unexposed treatment group (Fig. 5.3.3c GLM with treatment set as main effect, $F_{(1,202)} = 16.8$, $\eta_p^2 = 0.077$, $p = <0.001$. Extended data table 5.3.3.1c). Specifically, bumblebees in the 100 μM, 500 μM, and 1000 μM group consumed less on average than bees not exposed to caffeine over 3 days (Extended data table 5.3.3.1c). However, note that on average, caffeine-exposed bees did not consume less than their specific sucrose control group (Fig. 5.3.3a & 5.3.3c. Extended data table 5.3.3.1c).

The total dose that the bees consumed over the course of the experiment was also measured (Dose table 5.3.3). The dose of caffeine among the pre-exposed bees was as much as 9-84x greater than the lowest concentration of caffeine, depending on the treatment (Welch's ANOVA, $F_{(3,34.1)} = 93.0$, $\eta_p^2 = 0.761$, $p = <0.001$. Dose table 5.3.3a. Extended data table 5.3.3.2a). Unlike honeybees, bumblebees did not consume a significantly smaller dose of caffeine than the unexposed bumblebees during the 24 h choice test for any caffeine concentration (GzLM, concentration x treatment, $\chi^2_{(3)} = 0.693$, $p = 0.875$. Extended data table 5.3.3.2b). In all cases, the dose consumed by bees during

Bumblebees: Three day pre-exposure to caffeine in 0.5 M sucrose

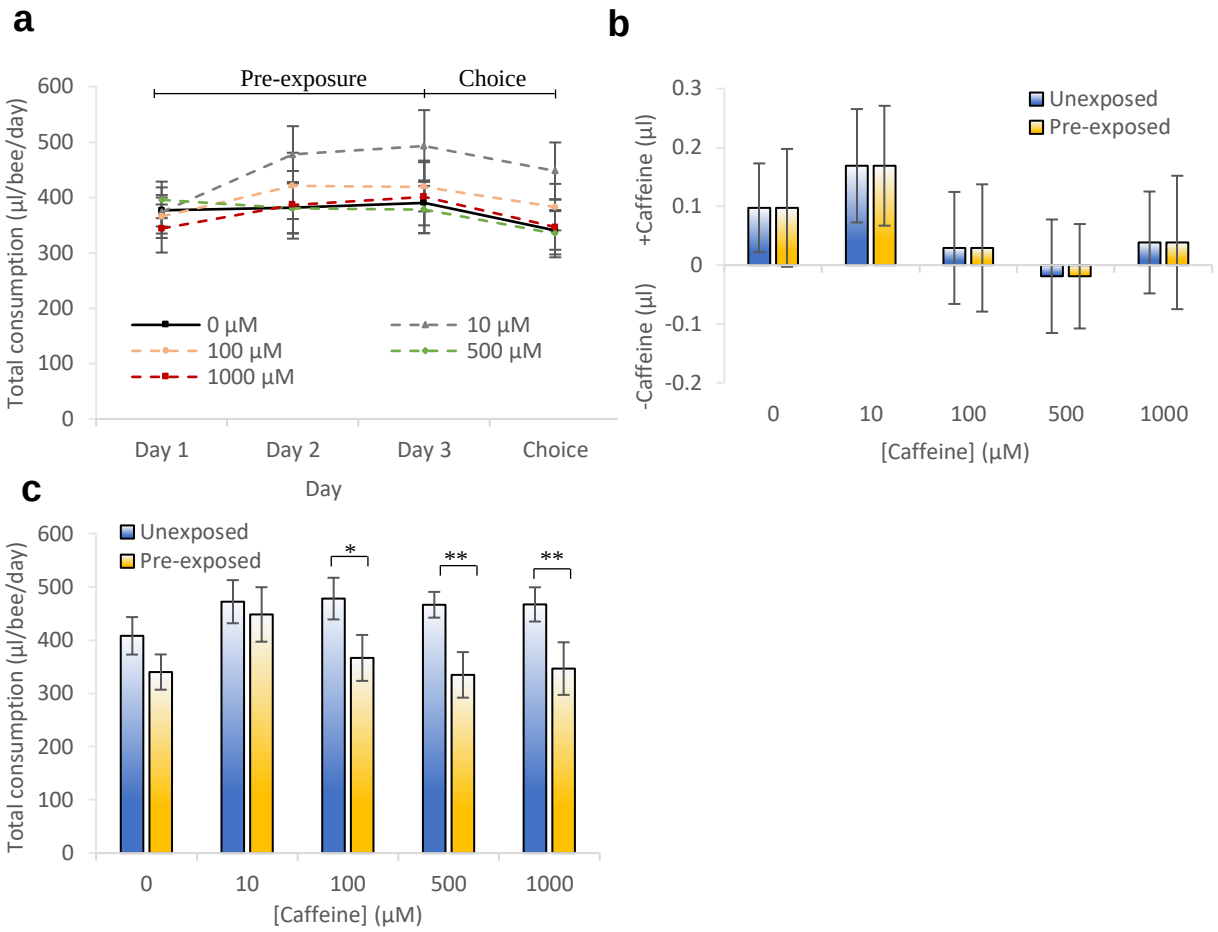

Figure 5.3.3 | Unexposed and pre-exposed bumblebees did not display a preference for caffeine when presented in 0.5 M sucrose. (a) Daily total consumption of pre-exposed bumblebees across the 3 day pre-exposure and 24 h two-way choice period. Total consumption did not vary across time (Extended data table 4a). **(b)** Neither unexposed or pre-exposed bumblebees displayed a preference for any caffeine concentration in a 24 h two-way choice (Extended data table 5.3.3.1b). Data represent the mean difference in the amount consumed over 24 h; positive values indicate a preference for caffeine and negative avoidance of caffeine. **(c)** Total volume consumed over the 24 h two-way choice day for unexposed and pre-exposed bumblebees. Pre-exposed bumblebees consumed a significantly lower total volume of 500 and 1000 µM caffeine in comparison to unexposed bumblebees (GLM with treatment and concentration set as main effects. $F_{(1,200)} = 14.1$, $\eta_p^2 = 0.066$, $p = < 0.001$), despite this, neither unexposed or pre-exposed bees differed from their respective controls (0 µM) (Extended data table 5.3.3.1c). Bars and line graphs indicate mean (±s.e.m.) of total consumption (µl) per bee, per day, controlled for by evaporation. Asterisks indicate significant differences for pairwise comparisons (Bonferroni) between treatments at each concentration (**p <0.01).

the choice period was less than the dose of caffeine consumed during the 3 day pre-exposure period (Dose table 5.3.3 Extended data table 5.3.3b). For example, bees pre-exposed to 100 μM caffeine consumed a dose of 7.80 μg/bee/day, 95 % CI [6.17, 9.44] during the pre-exposure period, but during the test, they chose a dose of 3.57 μg/bee/day, 95 % CI [2.40, 4.75] (*Post hoc* LSD between treatments, p = <0.001).

Mortality was not significantly different to controls in either the unexposed or pre-exposed bumblebees (lreg, $\chi^2_{(4)} = 6.35$, $p = 0.175$, and $\chi^2_{(4)} = 3.72$, $p = 0.455$, respectively).

Extended data table 5.3.3.1 | *B.terrestris* statistics for 24 h and 3 day chronic two-way choice in 0.5 M sucrose data. Data correspond to figure 5.3.3a-c **(a)** RM-GLM for the total consumption across the 3 day pre-exposure and 24 h two-way choice day period. n = 0 μM (19), 10 μM (21), 100 μM (21), 500 μM (20), 1000 μM (21). **(b)** GLM for the indexed data for the 24 h and 3 day pre-exposed two-way choice. n = unexposed 24 h: 0 μM (11), 10 μM (22), 100 μM (21), 500 μM (22), 1000 μM (21); Pre-exposed bees 0 μM (20), 10 μM (21), 100 μM (22), 500 μM (20), 1000 μM (21). **(c)** GLM for the 24 h two-way choice day total consumption for the unexposed and pre-exposed treatments and One-Way AONVAs for each treatment.

(a)†	Time: $F_{(3, 291)} = 2.55$, $\eta_p^2 = 0.026$, $p = 0.056$ Concentration: $F_{(4, 97)} = 0.845$, $\eta_p^2 = 0.034$, $p = 0.495$ Time x Concentration: $F_{(12, 291)} = 0.792$, $\eta_p^2 = 0.032$, $p = 0.659$
(b)	Treatment: $F_{(1, 202)} = 0.158$, $\eta_p^2 = 0.001$, $p = 0.691$ Concentration: $F_{(4, 202)} = 0.827$, $\eta_p^2 = 0.016$, $p = 0.510$ Treatment x Concentration: $F_{(4, 202)} = 0.238$, $\eta_p^2 = 0.005$, $p = 0.916$ One sample T-tests against zero (LSD): Unexposed 24 h two-way choice 0 μM $t_{(21)} = 1.29$ $p = 0.212$, 10 μM $t_{(21)} = 1.75$ $p = 0.094$, 100 μM $t_{(20)} = 0.307$ $p = 0.762$, 500 μM $t_{(21)} = -0.196$ $p = 0.847$, 1000 μM $t_{(20)} = 0.455$ $p = 0.661$ 3 day 24 h two-way choice 0 μM $t_{(20)} = -0.389$ $p = 0.702$, 10 μM $t_{(21)} = 1.43$ $p = 0.169$, 100 μM $t_{(22)} = 0.531$ $p = 0.601$, 500 μM $t_{(20)} = -0.098$ $p = 0.923$, 1000 μM $t_{(21)} = 0.184$ $p = 0.856$
(c)††	Treatment: $F_{(1, 02)} = 16.8$, $\eta p2 = 0.077$, p = <0.001 Concentration: $F_{(4, 202)} = 1.13$, $\eta_p^2 = 0.022$, p = 0344 Treatment x Concentration: $F_{(4, 202)} = 0.929$, $\eta_p^2 = 0.018$, $p = 0.448$ *Post hoc* pairwise comparisons between treatments at each concentration (Bonferroni): 0 μM $p = 0.224$, 10 μM $p = 0.635$, 100 μM $p = 0.032$, 500 μM $p = 0.007$, 1000 μM $p = 0.010$. One-Way ANOVA for unexposed bees: $F_{(4, 103)} = 0.682$ $\eta_p^2 = 0.026$ $p = 0.606$ One-Way ANOVA for pre-exposed bees: $F_{(4, 99)} = 1.10$ $\eta_p^2 = 0.043$ $p = 0.359$

† Data was square-root transformed. Mauchly's test indicated that the assumption of sphericity has been violated ($\chi^2_{(5)} = 11.4$, $p = 0.043$), therefore Huynh-Feldt corrected tests are reported ($\varepsilon = 1.00$).
†† Data was square-root transformed.

Dose table 5.3.2 Summary of the average caffeine dose consumed by *B. terrestris* following consumption of caffeine in 0.5 M sucrose. Data correspond to figure 5.3.3a-c. Values represent the average caffeine dose consumed per individual bee (µg/bee/day) and their respective 95 % CIs.

[Caffeine] µM	Pre-exposure period dose		Chosen dose			
			Pre-exposed		Unexposed	
	µg/bee/day	95 % CI	µg/bee/day	95 % CI	µg/bee/day	95 % CI
10	0.870	0.707, 1.03	0.476	0.367, 0.586	0.552	0.446, 0.657
100	7.80	6.17, 9.44	3.57	2.40, 4.75	5.14	3.96, 6.32
500	37.3	30.7, 44.0	16.1	10.7, 21.5	22.6	18.0, 27.3
1000	73.2	52.9, 93.6	36.5	21.5, 51.4	47.8	40.1, 55.5

Extended data table 5.3.3.2 | *B. terrestris* dosage statistics for 24 h and 3 day pre-exposure to caffeine. Data correspond to figure 5.3.3a-c and dose table 5.3.3. Welch's ANOVA for **(a)** The average dose consumed across the 3 day pre-exposure period **(b)** dose chosen during the unexposed 24 h two-way choice, and **(c)** Dose chosen during the 24 h choice by pre-exposed bees. *Post hoc* values are all possible pairwise comparisons between each concentration, Games-Howell adjusted. **(d)** GzLM for the chosen dose during the 24 h two-way choice between unexposed and pre-exposed bees. **(e)** GzLM for average dose consumed during the pre-exposure period and the dose chosen by pre-exposed bees during the choice test.

(a) $F_{(3, 34.1)} = 93.0$ $\eta_p^2 = 0.761$ $p = <0.001$. All *post hoc* value comparisons $p = <0.001$.

(b) $F_{(3, 35.0)} = 59.6$ $\eta_p^2 = 0.583$ $p = <0.001$. 500 µM vs 1000 µM $p = 0.085$. All other *post hoc* value comparisons $p = <0.001$.

(c) $F_{(3, 34.5)} = 169$ $\eta_p^2 = 0.756$ $p = <0.001$. 500 µM vs 1000 µM $p = 0.025$. All *post hoc* value comparisons $p = <0.001$.

(d) Concentration: $\chi^2_{(3)} = 1080$ $p = <0.001$

Treatment: $\chi^2_{(1)} = 7.54$ $p = 0.006$

Concentration x treatment: $\chi^2_{(3)} = 0.693$ $p = 0.875$

Post hoc comparisons between treatments at each concentration (LSD):

10 µM $p = 0.471$, 100 µM $p = 0.082$, 500 µM $p = 0.102$, 1000 µM $p = 0.195$.

(e) Concentration: $\chi^2_{(3)} = 1030$ $p = <0.001$

Treatment: $\chi^2_{(1)} = 48.9$ $p = <0.001$

Concentration x treatment: $\chi^2_{(3)} = 0.739$ $p = 0.864$

Post hoc comparisons between treatments at each concentration (LSD):

10 µM $p = 0.007$, 100 µM $p = <0.001$, 500 µM $p = <0.001$, 1000 µM $p = 0.002$.

5.3.4 The effect of increasing the sucrose concentration to 1.0 M sucrose on the bumblebee's preference for caffeine

It is possible that the concentration of the sucrose solution could influence a bee's preference for caffeine. To test this, bumblebees were fed with caffeinated solutions in 1.0 M sucrose for 3 days and then tested in a 24 h two-way choice assay. Pre-exposed bumblebees were fed with one of 4 treatments: 1.0 M sucrose (control), 10 μM, 25 μM, or 100 μM caffeine in 1.0 M sucrose for 3 days. As before, bumblebees that were provided with caffeine during the pre-exposure period did not consume more or less food than bumblebees that consumed sucrose alone (Fig. 5.3.4a. RM-GLM, treatment x concentration, $F_{(8.82,376)} = 1.57$, $\eta_p^2 = 0.035$, $p = 0.126$. Extended data table 5.3.4a). However, bumblebees were shown to decrease the total volume that they consumed during the course of the pre-exposure period (Time: $F_{(2.94, 376)} = 6.05$, $\eta_p^2 = 0.045$, $p = <0.001$), with all bumblebees consuming a lower total volume of food during the choice day in comparison to the first day of the pre-exposure period (*post hoc* Bonferroni, $p = <0.001$. Extended data table 5.3.4.1a). Note also that bumblebees fed with 1.0 M sucrose ate approximately 100-150 μl less total solution over the choice day, opposed to 0.5 M sucrose pre-exposed bees (Fig 5.3.4a).

When fed with 1.0 M sucrose solutions, bumblebees in general, did not prefer the caffeinated solution (Fig 5.3.4b. GLM, concentration x treatment, $F_{(3, 208)} = 2.04$, $\eta_p^2 = 0.029$, $p = 0.110$. Extended data table 5.3.4.1b). However, bees pre-exposed to 100 μM caffeine displayed a preference for the caffeinated solution as opposed to bees without prior caffeine exposure (Fig 5.3.4.1b. *Post hoc* Bonferroni, $p = 0.009$. Extended data table 5.3.4.1b).

As seen for bees fed with the 0.5 M sucrose solutions, bumblebees fed with caffeinated 1.0 M sucrose solutions consumed less total solution than the unexposed treatment group during the 24 h choice test (Fig. 5.3.4c. GLM with treatment set as main effect, $F_{(1,208)} = 37.0$, $\eta_p^2 = 0.151$, $p = <0.001$). Despite this, neither the pre-exposed bumblebees or unexposed bees differed from their specific sucrose-only control (0 μM) (Extended data table 5.3.4.1c).

Bumblebees: Three day pre-exposure to caffeine in 1.0 M sucrose

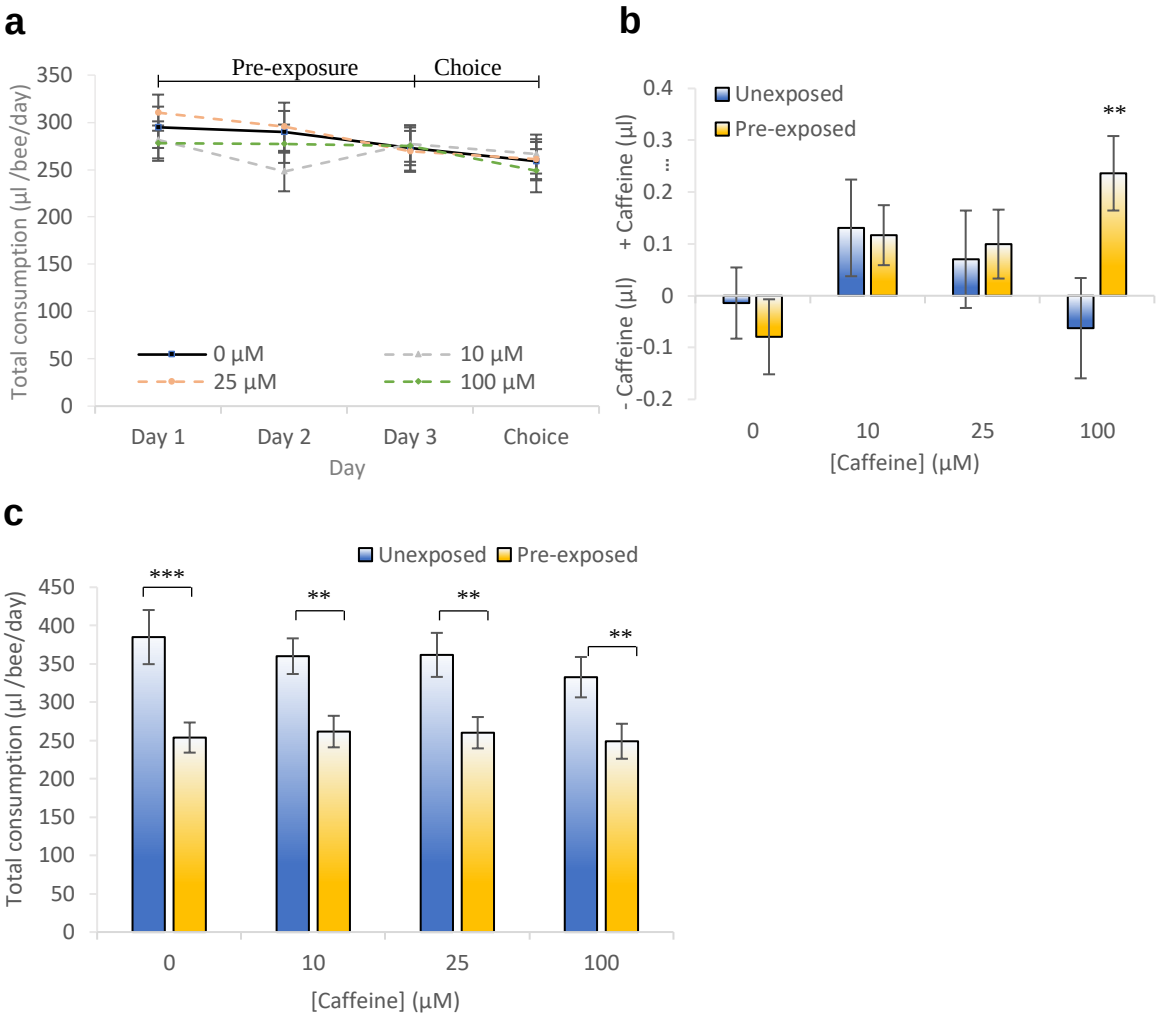

Figure 5.3.4 | Bumblebees displayed a preference for 100 µM caffeine following pre-exposure in 1.0 M sucrose. (a) Daily total consumption of pre-exposed bumblebees across the 3 day pre-exposure and 24 h two-way choice period. The inclusion of caffeine did not impact the total volume consumed (Extended data table 5.3.4.1a). **(b)** Unexposed bumblebees do not display a preference for any caffeine concentration in a two-way choice, however following pre-exposure, bumblebees significantly prefer 100 µM caffeine (Extended data table 5.3.4.1b). Data represent the mean difference in the amount consumed over 24 h; positive values indicate a preference for caffeine and negative avoidance of caffeine. Asterisks indicate significant differences for one-sample T-tests against 0 (**p <0.01) **(c)** Total volume consumed over the 24 h two-way choice day for unexposed and pre-exposure bumblebees. Pre-exposed bumblebees consumed significantly less than unexposed bumblebees at all caffeine concentrations (GLM with treatment set as main effect, $F_{(1,200)} = 35.5$, $\eta_p^2 = 0.151$, $p = < 0.001$. Extended data table 5.3.4.1c), despite this, neither unexposed or pre-exposed bees differed from their respective controls (0 µM) (Extended data table 5.4.3.1c). Bars and line graphs indicate mean (±s.e.m.) of total consumption (µl) per bee, per day, controlled for by evaporation. Asterisks indicate significant differences for pairwise comparisons (Bonferroni) between treatments at each concentration (**p <0.01).

The total dose that the bees consumed over the course of the experiment was also measured (Dose table 5.3.4). The dose of caffeine among the pre-exposed bees was as much as 3-10x greater than the lowest concentration of caffeine, depending on the treatment (Welch's ANOVA, $F_{(2,55.2)} = 250$, $\eta_p^2 = 0.862$, $p = <0.001$. Dose table 5.3.4. Extended data table 5.3.4.2a). Similar to the 0.5 M bumblebee experiments, pre-exposed bumblebees did not consume a significantly smaller dose of caffeine than the unexposed bumblebees during the 24 h choice test for any caffeine concentration (GzLM, concentration x treatment, $\chi^2_{(2)} = 1.72$, $p = 0.424$. Extended data table 5.3.4.2b). For instance, unexposed bumblebees chose a dose of 3.00 μg/bee/day, 95 % CI [2.32, 3.61], whereas pre-exposed bees chose a dose of 2.90 μg/bee/day, 95 % CI [2.26, 3.53] (*Post hoc* LSD between treatments, $p = 0.894$). In all cases, the bees consumed less caffeine than they were exposed to during the 3 day pre-exposure period (Dose table 5.3.4. Extended data table 5.3.4.2b). For example, bees pre-exposed to 100 μM caffeine consumed 5.37 μg/bee/day, 95 % CI [4.74, 6.01], during the pre-exposure period, but during the test, they selected a dose of 2.90 μg/bee/day, 95 % CI [2.26, 3.53] (*Post hoc* LSD between treatments, $p = <0.001$).

No unexposed bees died during the 24 h two-way choice for any treatment group. Mortality was not significantly different to the control for the pre-exposed bumblebees (lreg, $\chi^2_{(3)} = 6.64$, $p = 0.131$).

Extended data table 5.3.4.1 | *B.terrestris* statistics for 24 h and 3 day chronic two-way choice in 1.0 M sucrose data. Data correspond to figure 5.4.3. **(a)** RM-GLM for the total consumption across the 3 day pre-exposure and 24 h two-way choice day period. n = 0 μM (33), 10 μM (34), 25 μM (34), 100 μM (31). **(b)** GLM for the indexed data for the 24 h and 3 day pre-exposed two-way choice. n = unexposed 24 h: 0 μM (17), 10 μM (20), 25 μM (20), 100 μM (19); Pre-exposed bees: 0 μM (35), 10 μM (35), 25 μM (35), 100 μM (35). **(c)** GLM for the 24 h two-way choice day total consumption for the unexposed and pre-exposed treatments.

(a)†	Time: $F_{(2.94, 376)} = 6.05$, $\eta_p^2 = 0.045$, $p = <0.001$ Concentration: $F_{(3, 128)} = 0.134$, $\eta_p^2 = 0.003$, $p = 0.940$ Time x Concentration: $F_{(8.82, 376)} = 1.57$, $\eta_p^2 = 0.035$, $p = 0.126$ *Post hoc* comparisons for main effect of time (Bonferroni): day 1 vs choice day $p = <0.001$. All other pairwise comparisons $p = >0.05$.
(b)	Treatment: $F_{(1, 208)} = 0.121$, $\eta_p^2 = 0.006$, $p = 0.273$ Concentration: $F_{(3, 208)} = 1.67$, $\eta_p^2 = 0.024$, $p = 0.174$ Treatment x Concentration: $F_{(3, 208)} = 2.04$, $\eta_p^2 = 0.029$, $p = 0.110$ Pairwise comparisons between treatments at each concentration (Bonferroni): 0 μM $p =$ 0.580, 10 μM $p = 0.900$, 25 μM $p = 0.793$ 100 μM $p =0.009$ One sample T-tests against zero: Unexposed 24 h two-way choice: 0 μM $t_{(16)} = -0.205$ $p = 0.840$, 10 μM $t_{(19)} = 1.40$ $p = 0.176$, 25 μM $t_{(19)} = 0.749$ $p = 0.463$, 100 μM $t_{(18)} = -0.647$ $p = 0.526$ 3 day 24 h two-way choice: 0 μM $t_{(34)} = -1.10$ $p = 0.281$, 10 μM $t_{(34)} = 2.02$ $p = 0.051$, 25 μM $t_{(34)} = 1.50$ $p = 0.143$, 100 μM $t_{(34)} = 3.29$ $p = 0.002$ Cohen's d = 0.556.
(c) ††	Treatment: $F_{(1, 208)} = 37.0$, $\eta p2 = 0.151$, $p = <0.001$ Concentration: $F_{(3, 208)} = 0.483$, $\eta_p^2 = 0.007$, $p = 0.695$ Treatment x Concentration: $F_{(3, 208)} = 0.163$, $\eta_p^2 = 0.002$, $p = 0.921$ *Post hoc* pairwise comparisons between treatments at each concentration (Bonferroni): 0 μM $p = <0.001$, 19 μM $p = 0.004$, 25 μM $p = 0.007$, 100 μM $p = 0.003$. One-Way ANOVA for unexposed bees: $F_{(3, 136)} = 0.078$ $\eta_p^2 = 0.002$ $p = 0.972$ One-Way ANOVA for pre-exposed bees: $F_{(3, 72)} = 0.544$ $\eta_p^2 = 0.022$ $p = 0.654$

† Data was square-root transformed. Mauchly's test indicated that the assumption of sphericity has been violated ($\chi^2_{(5)} = 15.4$, $p = 0.009$), therefore Huynh-Feldt corrected tests are reported ($\varepsilon = 0.980$).
†† Data was square-root transformed

Dose table 5.3.4 | Summary of the average dose consumed by *B. terrestris* following consumption of caffeine in the 24 h two-way choice periods and during the pre-exposure period. Data correspond to figure 5.3.4a-c. Values represent the average caffeine dose consumed per individual bumblebee (μg/bee/day) and their respective 95 % CIs. Green indicates the dose where preference is observed.

[Caffeine] μM	Pre-exposure period dose		Chosen dose			
			Pre-exposed		Unexposed	
	μg/bee/day	95 % CI	μg/bee/day	95 % CI	μg/bee/day	95 % CI
10	0.522	0.454, 0.591	0.296	0.230, 0.362	0.400	0.317, 0.483
25	1.42	1.24, 1.60	0.706	0.534, 0.877	0.950	0.702, 1.19
100	5.37	4.74, 6.01	**2.90**	2.26, 3.53	3.00	2.32, 3.61

Extended data table 5.4.3.2 | *B. terrestris* dosage statistics for 24 h and 3 day pre-exposure to caffeine. Data correspond to figure 5.3.4a-c and dose table 5.3.4. Welch's ANOVA for **(a)** The average dose consumed across the 3 day pre-exposure period **(b)** dose chosen during the unexposed 24 h two-way choice, and **(c)** Dose chosen during the 24 h choice by pre-exposed bees. *Post hoc* values are all possible pairwise comparisons between each concentration, Games-Howell adjusted. **(d)** GzLM for the chosen dose during the 24 h two-way choice between unexposed and pre-exposed bees. **(e)** GzLM for average dose consumed during the pre-exposure period and the dose chosen by pre-exposed bees during the choice test.

(a) $F_{(2, 55.2)} = 250$ $\eta_p^2 = 0.862$ $p = <0.001$. All *post hoc* value comparisons $p = <0.001$.

(b) $F_{(2, 27.4)} = 36.4$ $\eta_p^2 = 0.618$ $p = <0.001$. 10 μM vs 25 μM $p = 0.001$. All other *post hoc* value comparisons $p = <0.001$.

†(c) $F_{(2, 60.0)} = 75.6$ $\eta_p^2 = 0.647$ $p = <0.001$. All *post hoc* value comparisons $p = <0.001$

(d) Concentration: $\chi^2_{(2)} = 319$ $p = <0.001$

Treatment: $\chi^2_{(1)} = 4.40$ $p = 0.036$

Concentration x treatment: $\chi^2_{(2)} = 1.72$ $p = 0.424$

Post hoc comparisons between treatments at each concentration (LSD):

10 μM $p = 0.093$, 25 μM $p = 0.099$, 100 μM $p = 0.894$.

(e) Concentration: $\chi^2_{(2)} = 690$ $p = <0.001$

Treatment: $\chi^2_{(1)} = 76.4$ $p = <0.001$

Concentration x treatment: $\chi^2_{(2)} = 0.556$ $p = 0.757$

Post hoc comparisons between treatments at each concentration (LSD):

10 μM $p = <0.001$, 25 μM $p = <0.001$, 100 μM $p = <0.001$.

† Data was square-root transformed

5.4 Discussion

The results indicate that the schedule of drug reinforcement has a significant effect on whether a preference for caffeine is acquired by adult worker honeybees or forager bumblebees. Unexposed honeybees displayed an aversion for 5 μM caffeine. However, this aversion was reversed when honeybees consumed caffeine chronically for a period of 3 days, resulting in a preference for the compound at the same concentration. In contrast, honeybees that consumed caffeinated solutions following intermittent schedules of pre-exposure: 12 h intermittent (I12), 48 h intermittent(I48), or extended 48 h intermittent (EXT48) did not display a significant preference for the alkaloid.

In contrast to honeybees, unexposed bumblebees were shown to be indifferent to caffeine, regardless of whether the compound was administered in 0.5 M or 1.0 M sucrose. However, similar to honeybees, a significant preference was apparent when the bees had chronically consumed caffeine for a period of 3 days, with bumblebees displaying a significant preference for 100 μM caffeine administered in 1.0 M sucrose.

5.4.1. Honeybees and bumblebees differ in the total volume of food that they consume across the pre-exposure period - Caffeine does not appear to suppress feeding in either bee species

Honeybees in the 3 day pre-exposed and I48 treatments were shown to vary their total consumption across the pre-exposure period. Honeybees consumed a greater total volume of food on the first day, followed by a decline in consumption on the second day, after which the total consumption remained approximately equal over the remaining 2 days. Honeybees undergoing the EXT48 schedule of administration displayed a similar response to the 3 day pre-exposed and I48 honeybees during the first 3 days of pre-exposure; however, both the 25 μM and 100 μM caffeine treated bees were shown to increase the total volume of food consumed during the choice period, relative to the sucrose-only control (~60 μl in control bees, and ~90 μl in both caffeine treatments). The response observed during the first 3 days of pre-exposure across the 3 day pre-exposed, I48, and EXT48 schedules of administration are similar to that observed in honeybees that were pre-exposed to nicotine for a 3 day period (Chapter 3.0). As discussed in chapter 3.0, this response is typically observed in caged experiments in honeybees, and I hypothesise that

this arises due to the starvation and stress endured during capture and restraint. However, it is not currently clear why honeybees increase their total food consumption following the EXT48 schedule of administration compared to the control.

Honeybees were shown to decrease the total volume of food that they consumed in only a single assay. Honeybees that consumed 100 μM caffeine in the I12 schedule displayed depressed feeding to the sucrose-only control; however, this was only apparent at the 36 h time point (i.e. following the second period of caffeine presentation). Although rodent models have identified increased food consumption following both chronic (Sweeney et al. 2016) and intermittent (Correa et al. 2018) caffeine access, results are often contradictory, with separate studies identifying either a decrease (Park et al. 2015) or no change in consumption (Pettenuzzo et al. 2008), following chronic caffeine treatment. At present, no mechanism has yet been identified to account for altered food consumption in response to caffeine administration in mammals. Interestingly, no other schedules of administration resulted in depressed feeding behaviour in the honeybee. Given that depressed feeding was observed only transiently in a single assay indicates that caffeine consumption does not typically result in depressed feeding in the honeybee. This is in contrast to the earlier results obtained in honeybees that had consumed nicotine, where depressed feeding was observed following both chronic and intermittent nicotine exposure, indicating that different alkaloids differentially affect the honeybees feeding behaviour when bees are exposed to these compounds over a number of days.

Honeybees in the I12 schedule of administration again showed a similar response to that observed in honeybees that underwent the nicotine I12 schedule of administration (chapter 3.0), with honeybees consuming a lower total volume of food at night (9 am-9 pm coinciding with sucrose administration) and a greater total volume during the day (caffeine administration from 9 am-9 pm). As discussed in chapter 3.0, this is likely accounted for by the sleeping patterns of honeybees, with honeybees consuming less food during the night.

As observed in the previous chapter, bumblebees did not display the characteristic feeding response that is typically observed in the honeybee pre-exposure assays in either of the experiments conducted. As discussed earlier (4.4.1), this likely arises from the

different methods of bee collection, with honeybee collection taking considerably longer than bumblebee collection, resulting in a longer period of starvation and possibly stress prior to experiment initiation, resulting in increased feeding behaviour in the honeybee.

Bumblebees in the 1.0 M sucrose 3 day pre-exposure assay were shown to decrease the total volume of food that they consumed over the course of the experiment; however, this decrease was only small (36 μl, representing a 12 % decrease from the first day of pre-exposure to the choice day). Importantly, none of the bees consuming caffeinated sucrose differed from the sucrose-only control, indicating that the decline in consumption over time was not due to the presence of caffeine in the bees food. Indeed, caffeine was not shown to suppress bumblebee feeding in any of the assays conducted, even when bumblebees consumed caffeine concentrations as high as 1000 μM. These results are in contrast to earlier studies conducted in the bumblebee subspecies *Bombus terrestris dalmatinus*, where caffeine concentrations as low as 1 μM have been shown to suppress feeding during a 24 h two-way choice test, using methods identical to those used in this thesis (Tiedeken et al. 2014), suggesting that different subspecies of bumblebee are differentially affected by the compound. In addition, the results obtained here are in contrast to the previous results obtained for bumblebees pre-exposed to nicotine, where both unexposed and pre-exposed bumblebees were shown to decrease the total volume consumed over the choice period when they had consumed high concentrations of nicotine (1000 μM) delivered in 1.0 M sucrose. This indicates that, similar to the results obtained in honeybees, caffeine and nicotine differentially affect the bumblebees feeding behaviour.

Collectively, the data collected in this chapter indicate that honeybees exhibit only a transient decrease in feeding behaviour following 12 h intermittent (I12) access to 100 μM caffeine, and neither unexposed, chronically pre-exposed, or honeybees pre-exposed to caffeine in either the I48 or EXT48 schedules of administration display depressed feeding in response to consuming caffeinated solutions. Furthermore, both unexposed and chronically pre-exposed bumblebees do not exhibit depressed feeding in a caged environment within a laboratory in response to caffeine concentrations as high as 1000 μM. Therefore, low concentrations of caffeine do not typically depress feeding in honeybees or *Bombus terrestris audax.*

Caffeine is found in floral nectar at concentrations between 3 – 1100 μM (Kretschmar and Baumann, 1999; Wright et al. 2013; Prado et al. 2019). Although it may be beneficial to test honeybees with high concentrations of caffeine (e.g. 1000 μM) to see if more concentrated caffeine solutions suppress honeybee feeding, at present, the results obtained in this chapter suggest that at least low ecologically relevant concentrations of caffeine are unlikely to suppress feeding in either the honeybee or *B. terrestris audax* in the field.

5.4.2. Unexposed honeybees and bumblebees do not display a preference for caffeine

In agreement with mammalian studies (Griffiths et al. 1986; Tordoff et al. 2008), these experiments show that unexposed honeybees and bumblebees did not prefer to drink caffeinated solutions during a 24 h two-way choice when the solutions were presented in a caged environment. Furthermore, surprisingly, unexposed honeybees were shown to display an aversion to a 5 μM caffeinated solution in a 24 h two-way choice, whereas aversion was not observed for the higher caffeine concentrations used (discussed in detail later).

A number of studies assessing preferential consumption of caffeine in honeybees and bumblebees have been conducted previously (summarised in table 5.4). Tiedeken et al. (2014) identified that caged bumblebees (*B. terrestris dalmatinus*) did not display a preference for a range of caffeine concentrations (1-10000 μM) in a 24 h two-way choice, this is in agreement with the indifference observed for caffeine in caged bumblebees in this study, indicating that both subspecies of bumblebee do not prefer caffeine in a 24 h two-way choice assay. Preferences for caffeine in free-flying honeybees have been seen, however, in a two-choice assay. For example, Singaravelan et al. (2005) identified that free-flying honeybees presented with a choice between a sucrose solution or ~130 μM or ~515 μM caffeine in sucrose displayed a preference for the caffeinated solution. However, this assay was conducted over a relatively short period of time (1 h). Another study in free-flying bees performed by Couvillon et al. (2015) identified that free-flying honeybees foraging on a sucrose solution containing 130 μM caffeine for a period of 3 h exhibited a greater frequency of foraging opposed to bees that foraged on sucrose alone. They also were more likely to perform the waggle dance and to dance more frequently, indicating

Table 5.4 | Studies examining preferences for caffeine in two-way choice assays in honeybees and bumblebees. Preferences are listed in green, indifference in black, and aversion in red. Honeybee (HB), bumblebee (BB).

Spp.	Concentration	Method	Citation
HB	65, **130**, 260, **515**, **770**, **1030** μM all delivered in 0.6 M sucrose	1 h free-flight choice between caffeine in sucrose or equimolar sucrose.	Singaravelan et al. 2005
BB	1, 10, 100, **1000**, 10000 μM All delivered in 0.5 M sucrose.	Caged 24 h two-choice assay between each concentration of caffeine in sucrose equimolar sucrose.	Tiedeken et al. 2014
HB	**0.5**, **5**, 50 μM caffeine all delivered in 25 % sugar water (sugar water composition not provided)	2 h free-flight choice between caffeine in sugar water or equimolar sugar water.	Liao et al. 2017

that the caffeinated solutions were perceived as more rewarding to the honeybees (Von Frisch, 1967; Seeley, 1991). This suggests that either (i) the positive reinforcing properties of caffeine are only present in short access paradigms (e.g. 1-3 h), (ii) the methodology employed (caged versus free-flight) affects the expression of a preference for solutions containing caffeine, or (iii) the concentrations of caffeine used in this study are insufficient to generate a preference in the bee over a 24 h period. Indeed, previous studies exploring nicotine preferences in bumblebees have also identified that short access free-flight assays, in comparison to 24 h caged assays, can alter preferences for the drug. For instance, bumblebees foraging on artificial flowers within the laboratory were shown to display a preference for 12 μM nicotine (Barrachi et al. 2015), whereas caged bumblebees offered a choice over a 24 h period displayed no preference for an identical concentration of the drug (Palmer-Young et al. 2017). This suggests that the discrepancies between this study and that of Singaravelan et al. (2005) may be accounted for by the experimental paradigms used.

Alternatively, the concentrations of caffeine used may account for the apparent indifference observed in the honeybee study. Singaravelan et al. (2005) identified a preference for both ~130 μM and ~515 μM, but not ~260 μM caffeine in free-flying honeybees over a 1 h choice period. Given that preferences were observed for marginally higher concentrations (130 μM) than those used in this study (100 μM) suggests that the

concentrations used in this study may have been too low to generate preferential consumption of the compound. Future studies may instead look to broaden the range of caffeine concentrations used in caged honeybee assays within the laboratory.

5.4.3 Honeybees display an initial aversion for 5 μM caffeine, however, they display a preference following a period of pre-exposure

Surprisingly, unexposed honeybees were shown to display an aversion to the 5 μM caffeinated solution in a 24 h two-way choice, whereas aversion was not observed for the higher caffeine concentrations used. Although this result was unexpected, this data supports a recent study by Liao et al. (2017). Using a free-flying assay whereby foragers had the option between 0.5, 5, or 50 μM caffeine in sucrose, or sucrose alone, Liao et al. (2017) identified that honeybees avoided both the 0.5 μM and 5 μM solutions. Conversely, no preference or aversion was seen for the 50 μM solution. Therefore, it appears that low concentrations of caffeine are aversive to the honeybee.

Aversion to low concentrations of caffeine could perhaps be explained by specific adenosine receptor oligomeric configurations in the bee brain. For instance, the A_1-A_1 homodimer in mammals has been shown to respond diametrically in response to low and high caffeine concentrations (Gracia et al. 2013), such that low concentrations of caffeine facilitate adenosine agonism, whereas high concentrations impede adenosine agonism. Furthermore, agonism of A_1 adenosine receptors has been shown to reduce extracellular dopamine concentrations in the reward pathways of the mammalian brain (Wood et al. 1989; Ballarin et al. 1995; Okada et al. 1997; Quarta et al. 2004; Karcz-Kubicha et al. 2003) and is known to impair methamphetamine (Kavanagh et al. 2015), and cocaine (Hobson et al. 2013) self-administration. A similar configuration of adenosine receptors in the bee brain could perhaps account for the valance reversal observed following pre-exposure. i.e. low concentrations of caffeine consumed within a 24 h period could facilitate adenosine binding and thus be deemed aversive. Conversely, chronic consumption and the resulting accumulation of caffeine within the system would therefore lead to a preferential blockade of adenosinergic signalling. Indeed, such oligomeric configurations of receptors in insects are not implausible. Oligomeric complexes in insects, although considerably understudied in comparison to mammals, are known to exist (Stengl and Funk, 2013; Camiletti et al. 2016), and recent evidence for an A_{2A}-D_2 receptor has been observed in the nematode

Caenorhabditis elegans (Manalo and Medina, 2018), indicating that oligomerisation of adenosine receptors is likely conserved across phyla. Future studies focused on identifying whether adenosine receptor oligomers exist in the bee brain, and their functional response to different concentrations of caffeine may aid in elucidating the cause of this valence reversal.

5.4.4 Honeybees and bumblebee display a preference for caffeine following chronic pre-exposure, but honeybees do not display a preference following intermittent caffeine administration

Previous studies in mammals have identified that rodents only display a preference for caffeine following either a period of forced chronic pre-exposure (Deneau et al. 1969; Atkinson and Enslen, 1976; Vitiello and Woods, 1975; Vautrin et al. 2005) or forced intermittent access (alternate day access for a period of 6 days) to the compound (Vautrin et al. 2005). The results obtained in this chapter identified that a significant preference for caffeine was only present in honeybees chronically pre-exposed to 5 μM caffeine administered in 1.0 M sucrose for a period of 3 days. In contrast, both the I12 and I48 schedules of drug administration did not result in preferential consumption of caffeine in the honeybee. Although there was a weak preference observed for the 100 μM concentration following the EXT48 schedule of administration (Marginal p-value: One sample T-test against zero, $p = 0.09$), the sample size was small (n = 10). This indicates that further studies are required to identify if extended schedules may generate a preference for higher caffeine concentrations in the honeybee. These results are in contrast to the earlier results obtained for honeybees that were provided with nicotine at the same concentrations of caffeine used in this study, following an I12 and I48 schedule of administration (see chapter 3.0), where a significant preference was observed for the nicotine that depended on the schedule of administration. This indicates that different schedules of administration differentially affect the preferential consumption of alkaloids in honeybees.

In contrast to honeybees, bumblebees that were pre-exposed to caffeine for a period of 3 days displayed a preference for 100 μM caffeine presented in 1.0 M, but not 0.5 M sucrose. Given that caffeine is encountered in the range of ~3-250 μM within the nectar of

Citrus and *Coffea* spp. (Wright et al. 2013), suggests that caffeine may be able to modulate honeybee and bumblebee behaviour in the field.

Preferences for addictive compounds are thought to be mediated by both positive and negative reinforcement, such that positive reinforcement drives the motivation in the early stages of addiction, whereas negative reinforcement drives continued consumption following the development of dependence (Koob and Le Moal, 2008). In addition, tolerance (i.e. the diminished response to the drug following its repeated use) may contribute to preferential consumption after extended drug use due to specific neuroadaptations in the brain, which attenuate the drug's ability to function as a positive reinforcer (Quarta et al. 2004). In mammals, both tolerance and dependence are thought to be mediated by an increase in adenosine receptor expression (Kaplan et al. 1993; Shi et al. 1994; Quarta et al. 2004). Indeed, increased expression of adenosine receptors has been observed in mammals following as little as 3 days pre-exposure to the drug (Hawkins et al. 1988; Ramkumar et al. 1988; Daval et al. 1989; Fredholm, 1982; Johansson et al. 1997; Johansson et al. 1993; Shi et al. 1993: Shi et al. 1994; Svenningson et al. 1999; Ning et al. 2015; O'Neill et al. 2015), in line with the chronic pre-exposure period used in this study. The increase in adenosine receptor expression within the mammals' reward pathways renders the pathway less responsive to the dose of caffeine the animal has become accustomed to, resulting in an attenuation of both the positive effects of caffeine (e.g. wake-promoting) (Griffiths and Mumford, 1996) and reinforcing properties (i.e. dopamine release) (Quarta et al. 2004), of the drug. In addition, caffeine dependence may also be generated due to increases in adenosine synthesis (Conlay et al. 1997) and adenosine efflux (Brito et al. 2016), resulting in an increase in adenosinergic tone, which is likely to contribute to caffeine withdrawal symptoms (Ribeiro and Sebastiao, 2010).

Given that honeybees in this study only displayed a preference for caffeine following chronic pre-exposure and not intermittent access suggests that withdrawal, at least following intermittent pre-exposure to caffeine concentrations of 25 μM and 100 μM, does not play a strong role in mediating preferential caffeine consumption in the honeybee. However, it is unclear whether withdrawal mediates preferential consumption of caffeine in honeybees chronically pre-exposed to 5 μM, or bumblebees chronically pre-exposed to 100 μM caffeine at this time. i.e. chronically pre-exposed bees may experience a state of

withdrawal if they do not choose to consume nicotine during the choice day, which could explain their preferential consumption of the compound. In addition, given that unexposed bees did not display a preference for caffeine in 24 h two-choice tests, suggests that caffeine does not function as a strong positive reinforcer in the bumblebee, at least over this time period. Therefore, tolerance, i.e. increased consumption of caffeine to once again experience the positive reinforcing effects of the compound, is unlikely. Indeed, similar to the results obtained for nicotine in the earlier chapters, it appears that honeybees and bumblebees were not attempting to match the dose of caffeine they were accustomed to during the pre-exposure period. For instance, honeybees were shown to consume a dose of 70.2/ng/bee/day when pre-exposed to 5 μM caffeine, however, they selected a dose of 43.6 ng/bee/day during the choice period. In addition, bumblebees were shown to consume a dose of 7.80 μg/bee/day when pre-exposed to 100 μM caffeine, however, they chose a dose of just 3.57 μg/bee/day during the choice period. This suggests that bees have not become tolerant to the effects of caffeine, i.e. if bees had become tolerant to the caffeine dose that they consumed over the pre-exposure period, then they would require an equivalent or higher concentration of caffeine during the choice period. Although tolerance to some of caffeine's effects (e.g. the wake-promoting effects of the compound) has been observed following pre-exposure in mammals (Griffiths and Mumford, 1996), the results obtained here are in agreement with two-choice assay studies conducted in rodents. For instance, mice that have been pre-exposed to 47 mg/kg caffeine dissolved in water for a period of 6 days display a preference for caffeine over water alone in subsequent two-choice tests; however, choose to consume a dose of just 27 mg/kg a day, rather than the high dose that they are accustomed to (Vautrin et al. 2005).

The preferential consumption of caffeine in both the honeybee and bumblebee following a period of forced pre-exposure is, therefore, most likely explained by the development of dependence, resulting in a requirement for the bee to continue caffeine use either to avoid withdrawal syndrome. Indeed, adenosine receptors are known to be expressed in the mushroom bodies of the honeybee brain (Naeger and Robinson, 2016; Kim et al. 2018), and their excitability (i.e. a heightened probability of firing an action potential) is increased following the application of low concentrations (100 μM) of caffeine (Wright et al. 2013). This increased excitability is thought to be specifically mediated by caffeine's interaction with adenosine receptors, as the specific adenosine receptor

antagonist Dipropylcyclopentylxanthine (DPCPX) similarly leads to an increase in KC excitability (Wright et al. 2013), indicating that caffeine interacts directly within the neuropiles responsible for encoding learning and memory of reward in the bee brain, as it does in mammals. In order to elucidate whether withdrawal is responsible for the preferential consumption of caffeine in the bee, future studies may focus on identifying whether behavioural elements of withdrawal are present following pre-exposure (e.g. deficits in learning and memory). In addition, analysis of changes in the critical components involved in adenosine signalling (e.g. adenosine receptor and adenosine transporter expression), as well as changes in haemolymph levels of adenosine, may aid in identifying the bees motivation to preferentially consume the drug.

Interestingly, honeybees did not display a preference for caffeine following any of the intermittent schedules of administration tested. Intermittent schedules of drug access have been found to lead to enhanced drug self-administration in comparison to long access alone. For instance, the motivation to self-administer cocaine is higher after intermittent access as opposed to long access, even though far less drug is consumed (Zimmer et al. 2012; Calipari et al. 2015), indicating the importance of temporal availability as opposed to the total drug dose received in generating an addictive state. Similar results have been observed in rodents for ethanol (Rosenwasser et al. 2013), heroin (Vendruscolo et al. 2011), and nicotine (Cohen et al. 2012). In addition, the number of intermittent periods of abstinence the animal undergoes, the greater the enhancement of drug self-administration (Skjei and Markou, 2003; Gilpin et al. 2014). Although intermittent paradigms have been tested in rodent models of caffeine addiction (alternate day access for a period of 6 days) (Vautrin et al. 2005), and have been shown to lead to a preference for the drug in a two-choice test (Vautrin et al. 2005), at present no studies have systematically assessed whether, similar to other drugs of abuse, intermittent caffeine administration results in enhanced self-administration of the drug in comparison to chronic access alone. Therefore, it is not currently clear if intermittent access schedules affect a preference for caffeine in animal models of addiction.

Indeed, the schedules of administration that result in enhanced drug-seeking in rodent models are highly specific. For instance, increased self-administration of nicotine requires 23 h nicotine access a day following by periods of 24-48 h drug abstinence (Cohen

et al. 2012), whereas 6 h nicotine access followed by 18 h abstinence does not result in escalated nicotine use (Paterson and Markou, 2004; Kenny and Markou, 2006). In comparison, 6 h cocaine access results in robust drug self-administration (Ahmed and Koob, 1999), whereas 23 h access a day does not (Carroll et al. 1989). Therefore, it may also be that the schedules of drug access used in this study are not suited for generating a preference for caffeine in the honeybee, and alternate temporal patterns of drug administration may prove more effective. Alternatively, it may simply be that intermittent schedules of caffeine pre-exposure may not be best suited to studying whether caffeine functions as an addictive agent in the honeybee, and chronic pre-exposure assays may prove more fruitful. Future studies may look to expand the range of caffeine administration protocols used to identify whether drug access is a factor in the honeybees preferential consumption of the compound.

5.4.5 Pre-exposure to caffeine does not affect the caffeine dose chosen in the test period

In both species, the preferred chosen dose following the pre-exposure period was not significantly different from the dose chosen by unexposed bees (Honeybees: unexposed chosen dose = 45.2 ng/bee/day, pre-exposed chosen dose = 43.6 ng/bee/day. Bumblebees: unexposed chosen dose = 3.00 μg/bee/day, pre-exposed chosen dose = 2.90 μg/bee/day). This likely arises from the fact that the total consumption in the unexposed 24 h choice periods was consistently greater than the total consumption on the choice periods following prior pre-exposure. Regardless, this indicates that identical caffeine doses are not preferred unless preceded with a period of 3 days pre-exposure, indicating that the pre-exposure period is critical in mediating preferential consumption of the drug.

5.4.6 Changing the concentration of sucrose that caffeine is administered in does not affect the preference for caffeine in the bumblebee

Previous studies have indicated that bees are less likely to reject alkaloids when presented in more concentrated sucrose solutions (Gegear et al. 2007; Köhler et al. 2012a), presumably, as an increased concentration of sucrose is more effective at masking the bitter taste of these compounds (Cocco and Glendinning, 2012; Köhler et al. 2012a). In order to assess whether sucrose molarity affected the preference for caffeine in caged bumblebees

during the 3 day pre-exposure period, experiments were repeated in both 0.5 M and 1.0 M sucrose. Although bees were shown to display a preference for 100 μM caffeine in 1.0 M sucrose (representing a chosen dose of 2.90 μg/bee/day), whereas no preference was observed for the same caffeine concentration in 0.5 M sucrose (representing a chosen dose of 3.57 μg/bee/day, subsequent analysis of both the 0.5 M and 1.0 M sucrose concentrations in combination did not show an effect of sucrose concentration on the preferential consumption of the drug (Appendix: Supplementary Fig. 3.1), indicating that the increase in sucrose concentration did not account for the preference observed. This is in agreement with both the previous honeybee and bumblebee study assessing the role of sucrose molarity on nicotine preference (discussed in 3.4.4 & 4.4.4), and instead suggests that the average dose consumed across the pre-exposure period is likely to account for the preferential consumption of caffeine in the higher molarity sucrose opposed to the change in vehicle molarity.

5.4.7 Conclusion

Pre-exposure to caffeinated solutions for three days leads to a preference for caffeine in subsequent two-choice tests in both the honeybee and bumblebee, indicating that caffeine preferences can be established in a manner analogous to that observed in mammals. However, this study, alongside the literature to date, indicates that the bees' response to caffeine is complex, and it is not currently clear why low concentrations of caffeine are aversive to honeybees.

Although there was a weak effect observed in the EXT48 intermittent pre-exposure on the subsequent choice behaviour of the honeybee, at present, it is not clear whether intermittent schedules of caffeine administration will prove fruitful to the study of caffeine addiction in insects and further studies are required to ascertain whether the EXT48 schedule or indeed alternate temporal schedules of administration are capable of generating caffeine preferences in the bee. However, the results from this study suggest that chronic pre-exposure paradigms may be best suited for future research. In addition, although both honeybees and bumblebees were shown to display a preference for caffeine following pre-exposure, at present, what accounts for this preferential consumption behaviour is unclear. Future studies may therefore look to ascertain whether bees experience symptoms of withdrawal following caffeine pre-exposure. In addition, molecular studies assessing

changes in gene expression of the critical components of adenosinergic signalling following caffeine pre-exposure would also aid in understanding the bees motivation to preferentially consume caffeine during the choice period.

As has been noted for the nicotine data collected in the two previous chapters, the data collected in this study is insufficient to characterise either the honeybee or bumblebee as displaying caffeine addiction in line with the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) or the World Health Organizations International Classification of Diseases (ICD10) criteria for addiction. Future studies may therefore look to assess specific symptoms in line with criteria, e.g. continued use despite harm and evidence of withdrawal.

Chapter 6.0 General discussion

The intention of this thesis was to examine whether honeybees and bumblebees could function as viable models of addiction for the alkaloids nicotine and caffeine in a caged setting within the laboratory. Specifically, it looked to assess the ideal species, concentration, and schedules of administration that would result in preferential consumption of these alkaloids.

6.1 Synthesis of nicotine studies in the honeybee and bumblebee.

Honeybees and bumblebees were shown to display a preference for nicotine that depended on the concentration and schedule of administration used. Unexposed honeybees and bumblebees did not display a preference for nicotine in a range of sucrose concentrations, and preferences were only observed following pre-exposure to the compound. Given that pre-exposure periods in rodent models increase nicotine self-administration by facilitating neurological changes in advance of the test period (Damaj et al. 2003; Grabus et al. 2005 Gould et al. 2012; Natividad et al. 2013; Renda and Nashmi, 2014), suggests that similar neurological changes are required in order to generate a preference for nicotine in the bee. Indeed, upregulation of nicotinic acetylcholine receptors (nAChRs) have been observed in the honeybee following 3 days pre-exposure to nicotine (Christen et al. 2016), and upregulation of nAChRs is known to contribute to the development of tolerance and dependence in the mammal (Buisson and Bertrand, 2002; McCallum et al. 2006; Nashmi et al. 2007; Tapper et al. 2007; Govind et al. 2009; Renda and Nashmi, 2014; Meyers et al. 2015; Brunzell et al. 2015). In addition, similar pre-exposure protocols have been shown to generate preferences for ethanol in other insects, where unexposed *Drosophila* are indifferent to ethanol; however, display a robust preference for the chemical following a single day of ethanol pre-exposure (Peru y Colón de Portugal et al. 2014). This, alongside the data collected in this thesis, suggests that pre-exposure protocols may be beneficial for assessing the viability of insects to study a range of addictive compounds.

Honeybees and bumblebees were shown to differ in the schedules of drug administration that led to preferential consumption of nicotine. Bumblebees were shown to display a preference for 100 μM nicotine delivered in a sucrose solution following three

or five days pre-exposure to the compound. In contrast, no preference was observed following three or five days pre-exposure in the honeybee. Rather, the honeybee only displayed a preference for nicotine following intermittent schedules of nicotine administration. The fact that intermittent periods of abstinence were needed in order to observe preferential consumption of nicotine in the honeybee is suggestive of negative reinforcement behaviour (George and Koob, 2017). In contrast, the fact that preferences for nicotine were observed in the bumblebee through chronic pre-exposure alone makes inferring the underlying motivation for preferential nicotine consumption difficult. Assessment of withdrawal symptomology in both species will ascertain whether negative reinforcement is contributing to the preferential nicotine consumption observed.

Rodent models of nicotine addiction have identified a number of factors that affect self-administration of the drug across rat strains. For instance, changes in the relative expression of specific nAChR subunits and dopamine receptors are known to alter preferential consumption of nicotine (Zhang et al. 2000; Gozen et al. 2016). In addition, rodents that display reduced cytochrome P450 (CyP) functionality, the enzymes responsible for nicotine metabolism, exhibit increased responsivity to the rewarding effects of nicotine (Li et al. 2013; Bagdas et al. 2014; Budzynska et al. 2016) and an increase in withdrawal intensity (Bagdas et al. 2014). Finally, rodent studies have repeatedly identified that the age of the animal is perhaps the single most important determinant of nicotine addiction susceptibility (Schramm-Sapyta et al. 2009; Buchmann et al. 2013). Adolescents rodents have been shown to be more responsive to nAChR upregulation (Trauth et al. 2001; Adriani et al. 2003; Levin et al. 2007; Renda et al. 2016; Yuan et al. 2015; Melroy-Greif et al. 2016), more sensitive to the rewarding effects of the drug (Belluzzi et al. 2004; Shram et al. 2006; Brielmaier et al. 2007; Torres et al. 2008), less susceptible to the negative effects of the drug (Elliott et al. 2004; Wilmouth and Spear, 2004; Shram et al. 2006), less susceptible to withdrawal symptoms (Natividad et al. 2010; Schramm-Sapyta et al. 2009; Natividad et al. 2013), and voluntarily self-administer as much as 3-fold increases in nicotine dose compared to adult rodents (Adriani et al. 2002b; Adriani et al. 2003; Levin et al. 2003; Levin et al. 2007; Levin et al. 2011; Natividad et al. 2013; Renda et al. 2016). This suggests that changes in the relative expression of target receptors, metabolism of the nicotine, or age may also account for the differences observed between the honeybee and bumblebees in this study.

Unfortunately, the relative expression of both nAChRs and dopamine receptors in the reward centres of the bee brain has not yet been systematically assessed across bee species. Therefore, it is not currently possible to identify if changes in the relative expression of these receptors may account for the differences observed between the honeybee and bumblebee in this study. However, genomic studies have identified that the honeybee and bumblebee differ in their number of CyP genes. Whereas the honeybee has three CyP genes responsible for clearance of xenobiotic compounds (e.g. nicotine or neonicotinoids), the bumblebee has only two (Mao et al. 2011; Sadd et al. 2015; Manjon et al. 2018). This reduction in CyP gene functionally has been shown to reduce the bumblebees ability to metabolise neonicotinoid pesticides in comparison to the honeybee (Cresswell et al. 2014) and renders the bumblebee more sensitive to the effects of neonicotinoids (Heard et al. 2017; Robinson et al. 2016; Manjon et al. 2018), resulting in lower LC50 for a range of neonicotinoids in comparison to the honeybee (Heard et al. 2017; Robinson et al. 2017; Manjon et al. 2018). Although specific analysis of nicotine sensitivity across bee species has yet to be conducted, given that reduced CyP functionality increases both the rewarding effects of nicotine and withdrawal intensity in rodent models of nicotine addiction, suggests that these changes in CyP function could, perhaps in part, explain the differences observed across bee species in this study.

It is important to note that the age of the bumblebee was not factored into this study. Unlike honeybees, where foraging status is dictated by the age of the bee, with workers transitioning to foraging once they reach 3 weeks of age (Johnson, 2010), bumblebees typically initiate foraging behaviour at a very young age. Indeed, bees as young as 2 days post-emergence are known to readily engage in foraging behaviour (Brian, 1952; Pouvreau, 1989; Yerushalmi et al. 2006; Tobback et al. 2011) and may forage their entire life, which can be as long as 70 days (Hagbery and Nieh, 2012). Therefore, there is a strong likelihood that the bumblebee foragers used in this study represent a heterogeneous group of both older and younger bees, in contrast to the foragers used in the honeybee study, which strictly represent adult insects.

Only two studies to date have assessed the effect of age on nAChR agonist preferences in invertebrates. Kessler et al. (2015) identified that newly emerged

bumblebees (i.e. < 24 h from emergence) avoided 1 and 10 nM IMD; however, worker bumblebees displayed a preference for 1 nM IMD in a 24 h two-way choice in a caged setting within the laboratory. In addition, newly emerged honeybees were shown to avoid 1 but not 10 nM IMD, whereas adult forager honeybees displayed a preference for 10 nM IMD using an identical assay (Kessler et al. 2015). In addition, a single study in the nematode has identified that newly hatched worms are indifferent to nicotine, whereas young adult worms exhibited a strong preference for the drug as exhibited by preferential chemotaxis to nicotine-containing regions in an agar plate (Sellings et al. 2013). Similar to mammals, preferences were shown to decline rapidly as the worm aged (Sellings et al. 2013). Given that newly emerged bees avoid and newly hatched worms are indifferent to nAChR agonists, whereas adolescent worms displayed a strong preference for the drug, suggests that the age of the bees used in this study may account for the differences in preferential nicotine consumption observed across species. Indeed, expression of nAChRs subunits in the honeybee brain is known to vary depending on the age of the insect (Jones et al. 2006). Future studies are therefore strongly recommended to control for the age of bee when assessing preferential consumption of nicotine.

6.2 How do bees discriminate between solutions during the choice period?

The bees used in these experiments were presented with a choice between a tube containing an alkaloid dissolved in a sucrose solution or a tube containing sucrose alone, in a caged environment in permanent darkness within an incubator. In order for the bees to successfully make a choice between either solution, they would need to form an association between the tube that contains the chosen solution and the location of that tube within the cage. Given that the bees were housed in a dark environment, with no other cues present, the bee must therefore rely on pre- or post-ingestive means to form this association, and therefore to locate their tube of choice throughout the 24 h choice period.

Honeybees and bumblebees express gustatory receptors in gustatory receptor neurons (GRNs) located within sensilla on their mouthparts, antennae, and tarsi (Simcock et al. 2017). Electrophysiological recordings have shown that honeybees can taste 10 mM caffeine dissolved in 300 mM sucrose (Wright et al. 2013) and 1 mM nicotine dissolved in water alone (Kessler et al. 2015) when the solutions are presented to the bees mouthparts. Similar results have been obtained in the buff-tailed bumblebee in response to nicotine,

where concentrations as low as 10 μM elicit spiking activity from GRNs in the mouthpart sensilla when delivered in a 100 mM sucrose solution (Unpublished data, Wright laboratory). It is not yet known whether honeybees can taste caffeine or nicotine at concentrations lower than 10 mM, or 1 mM, respectively. Nor is it known whether bumblebees can detect caffeine. In addition, it is not known whether dissolving these compounds in higher sucrose concentrations, such as the ones used in this thesis, will affect the bees ability to taste these alkaloids. Indeed, sugar solutions appear to suppress the activity of bitter-sensing gustatory neurons in response to some bitter compounds in *Drosophila* (French et al. 2015). Interestingly, however, this suppression is not apparent in response to nicotine or caffeine (French et al. 2015). Furthermore, bitter solutions can also suppress the activity of sugar-sensing neurons, impairing the insect's ability to detect sweet solutions (Jeong et al. 2013). Indeed, how these different taste modalities are encoded when presented in combination is not currently clear and appears to vary depending on the bitter and sweet substance in question and their relative concentrations (reviewed in French et al. 2015b). However, given that bees are known to contain gustatory receptor neurons that respond to both of these alkaloids, it is, therefore, possible that the bees used in these experiments could taste these alkaloids, and taste could possibly be used as a cue with which to discriminate between solutions during the choice period. In addition, bees utilise magnetoreception to detect the earth's magnetic field, which they use as an internal compass to orientate themselves within the darkness of the hive (Liang et al. 2016). In combination, this would allow the bee to discriminate between the different tubes during the choice period and to associate each tube with a specific location within the cage.

Alternatively, the bees may form an association with the location of their tube of choice and the post-ingestive consequences of consuming the solution within that tube. Consumed solutions are rapidly absorbed into the bee's haemolymph, where they make their way into the head capsule in at least 30 s (Simcock et al. 2018). This rapid absorption would allow any pharmacological effects of the alkaloids (i.e. nAChR desensitisation or adenosine receptor antagonism) to be associated with the location of the tube as the bee is consuming the solution, importantly, this should occur even in the absence of any taste cues (i.e. if more concentrated solutions were masking the taste of an alkaloid). Indeed, freely-moving bumblebees have been shown to spend as long as 70 s consuming sugar solutions in a single bout within a laboratory setting (Unpublished data, Wright laboratory),

providing plenty of time for the alkaloid to make its way into the brain capsule and exert its pharmacological effects. Furthermore, bees are known to form robust memories from post-ingestive effects of secondary metabolites alone when decoupled from their pre-ingestive detection by injecting the compound directly into the bee's haemolymph (Wright et al. 2010).

In conclusion, the bees used in this study could form an association between their chosen tube and its location within the cage using either pre- or post-ingestive means, in combination with their internal magnetic compass. However, it is not currently clear whether bees are relying on the taste of the alkaloid, the post-ingestive pharmacological effects of the alkaloid, or both pre- and post-ingestive mechanisms in combination, to identify their chosen solution during the choice period (see below). Future studies should therefore focus on identifying whether honeybees and bumblebees can taste nicotine and caffeine at the concentrations tested in this thesis and whether an increase in the sucrose concentration the alkaloids are administered in impacts their ability to discriminate between the solutions offered during the choice period. Taste discrimination methodologies exist in *Drosophila* that could be readily adapted for the bee (Maseka and Scott, 2010); this, in combination with electrophysiological studies, would shed light on the mechanism used.

As honeybees and bumblebees rely on visual cues, such as colour, when foraging naturally in the field (Chittka and Raine, 2006), and bees can very rapidly form an association between a colour cue and a rewarding solution in laboratory settings (Barrachi et al. 2017a), future studies may therefore use different coloured tubes in light conditions to make it easier for the bees to associate a tube with their solution of choice. Furthermore, previous studies using free-flight assays have identified that bumblebees remain faithful to flower colours that contain nicotine, over flower colours that contain sucrose alone, even when these flower colours become the sub-optimal choice, achieved by replacing the nicotine-sucrose solution with water (Barrachi et al. 2017a). By allowing the bees to form colour associations between solutions in caged assays, memory extinction experiments, such as those performed in free-flight assays, could also be incorporated into future studies, e.g. by changing the coloured tube previously associated with an alkaloid to water or

sucrose alone, the persistence of the bees memory could be tested simultaneously (Barrachi et al. 2017a).

6.3 What indication is there that honeybees and bumblebees display a biphasic dose-response curve to caffeine and nicotine in 24 h two-choice tests?

As detailed in section 1.5.2, animals typically display a biphasic (inverted U-shape), or a J-shaped response curve, depending on the end-point used, when offered a choice to self-administer an addictive compound (Koob and LeMoal, 2006). This biphasic response is thought to arise as low concentrations of the compound are insufficient to activate reward circuitry, whereas high concentrations may lead to unwanted side-effects, such as malaise, and are therefore avoided (Koob and LeMoal, 2006; Calabrese, 2008). This leaves a narrow dose range in which animals will reliably self-administer a drug. It is important to note that in the mammalian literature, dose can mean either the *relative dose* (the dose provided is relative to a particular property of the subject, typically the concentration is scaled to the subjects weight (e.g. 0.5 mg/kg/infusion) hence the term 'dose' is used instead of 'concentration') and the *absolute dose* (the total amount of the compound administered to a subject over the entire experiment). The relative dose, opposed to the absolute dose, is the dose presented in dose-response curves and is synonymous with the term concentration.

There is little evidence of a clear biphasic response in any of the experiments conducted in this thesis. Weak evidence of a biphasic response is present in bumblebees pre-exposed to nicotine for a period of 3 days in 1.0 M sucrose (Fig 4.3.2b) and in honeybees following the nicotine intermittent 48 schedule of administration (Fig 3.3.4c). However, in the latter, only two nicotine concentrations are used, which is insufficient to accurately assess a biphasic response. Although there may be evidence of a J-shaped response in bumblebees chronically pre-exposed to caffeine for 3 days in 1.0 M sucrose (Fig 5.3.4b), and again in honeybees exposed to the nicotine intermittent 12 schedule (Fig 3.3.4c), collectively, there is insufficient evidence that suggests that biphasic or J-shaped responses are present in honeybee and bumblebees responses to nicotine and caffeine.

The concentrations of caffeine and nicotine used in this thesis were chosen based on the concentrations of nicotine and caffeine found naturally in the floral rewards of plants

and on the literature to date, which has assessed preferential consumption of these alkaloids in bees, which have largely focussed on ecologically relevant concentrations. As detailed in Table 1.5.3 and 5.4, at present, there is no consensus in the literature as to what concentrations or methodologies result in avoidance, indifference, or a preference for caffeine and nicotine in bumblebees and honeybees. As discussed throughout this thesis, this may be accounted for by the species or subspecies used, as well as the methodology employed (e.g. duration and method of exposure). Although 10-fold serial dilutions are often used in the literature when assessing the bee's responsivity to alkaloids (e.g. Wright et al. 2013; Tiedeken et al. 2014; Liao et al. 2017), this approach was specifically avoided in this thesis. Although 10-fold serial dilutions allow for easy comparison between concentrations, they are not conducive to identifying whether a biphasic dose-response is present. i.e. the concentration range is simply too large to identify a narrow dose range. For this reason, concentrations were continuously assessed throughout, and changes made to try and narrow down if and at what concentration bees prefer either alkaloid. Although clear biphasic responses are not observed, this does not mean that they are not present, and the concentrations used in this thesis may simply lack the resolution to observe a biphasic response curve. e.g. although bumblebees only display a preference for 100 μM in 1.0 M sucrose, and not in response to 25 or 500 μM, if a narrower concentration series is used, e.g. 0, 25, 50, 75, 100, 125, 150, and 175 μM nicotine, then a biphasic response may be observed. Future studies may therefore repeat the studies conducted in this thesis using a narrower range of alkaloid concentrations, based on the alkaloid concentrations that honeybees and bumblebees preferentially consume in this thesis, to see if biphasic responses are present.

6.4 The sucrose concentration an alkaloid is administered in does not affect a preference for nicotine or caffeine

Previous studies have identified that the bees aversion to secondary metabolites can be offset by increasing the sucrose concentration that the compound is administered in (Gegear et al. 2007; Köhler et al. 2012a; Wright et al. 2013). For example, free-flying bumblebees offered a choice between 1.0 M sucrose alone and 1.0 M sucrose containing 155 μM gelsemine in artificial flowers, showed a strong aversion for flowers containing gelsemine (Gaeger et al. 2007). However, when offered a choice between artificial flowers

containing 1.0 M sucrose or 2.0 M sucrose containing the same concentration of gelsemine, bumblebees foraged on both flowers at equivalent rates (Gaeger et al. 2007). This indicates that bees are willing to consume bitter chemicals when these chemicals are administered in a higher molarity sucrose solution. However, it is not clear in this instance whether bees are foraging at equivalent rates because the higher molarity sucrose masks the taste of gelsemine, or if bees could still taste the compound but were simply willing to overcome the bitter taste in order to secure a more rewarding solution. Köhler et al. (2012a) identified that free-flying honeybees presented with a 10-way choice between a range of nicotine concentrations (0, 3, 6, 15, 30, 60, 150, 300, 500, and 1000 μM nicotine) delivered in 0.15 M sucrose, found 30 μM nicotine aversive. In comparison, bees presented with an identical 10-way choice with nicotine presented in 0.3 M sucrose only displayed an aversion to nicotine at concentrations ≥ 150 μM (Köhler et al. 2012a). This indicates that free-flying honeybee's aversion to nicotine can be offset by increasing the concentration of the sucrose the compound is administered in. However, interestingly, bees presented with an identical 10-way choice with nicotine presented in 0.6 M sucrose also only displayed an aversion to nicotine at concentrations ≥ 150 μM. That is, the ability of a higher molarity sucrose concentration to offset an aversion to nicotine is identical when the sucrose concentration is 0.3 M and 0.6 M, and increasing the sucrose molarity concentration further does not result in a greater offset in the honeybees aversion for nicotine. This suggests that there is a ceiling effect for sucrose to mask the taste of high concentrations of nicotine in the honeybee.

Similar results have been identified for caffeine using the proboscis extension response in harnessed honeybees (Wright et al. 2013). Here, the proportion of bees that extended their proboscis following stimulation with a caffeinated sucrose solution was tested. Honeybees were shown to reduce their rate of proboscis extension to 100 μM caffeine when the alkaloid was delivered in 0.3 M sucrose, indicating that they found this solution aversive. In contrast, when caffeine was delivered in 0.7 M or 1.0 M sucrose, aversion was only apparent at 1000 μM caffeine. Therefore, increasing the sucrose concentration by 0.4 M offset the honeybee's aversion to 100 μM caffeine. However, there was no difference between the honeybee's aversion to 1000 μM caffeine when delivered in 0.7 M or 1.0 M sucrose, despite a 0.3 M difference in sucrose concentration. This again suggests that there is a ceiling effect, where increasing the sucrose molarity concentration

further does not result in a greater offset in the honeybee's aversion to caffeine. Indeed, although mammalian taste receptors are molecularly distinct from insect taste receptors (Yarmolinsky et al. 2009), ceiling effects have also been observed in compounds designed to mask the bitter taste of caffeine in human foods, whereby low concentrations of a masking compound may mask the taste of caffeine, however, increasing the concentration of a masking compound further does not result in a greater ability to offset the bitter taste of the caffeine (Ley, 2008). As discussed above, it is not currently clear how different taste modalities (i.e. bitter and sweet) are encoded when presented in combination in insects; therefore, further studies (e.g. taste discrimination and electrophysiological studies) are required to ascertain whether there is a ceiling effect for sucrose to mask the bitter taste of caffeine and nicotine in the bee.

The concentrations of sucrose used in this thesis were 0.5 M, 1.0 M, and 1.5 M, depending on the alkaloid and the experiment in question. However, in all instances, increasing the concentration of sucrose did not affect the honeybee or bumblebees consumption of either nicotine or caffeine in a 24 h two-choice test (appendix; Supplementary Fig. 1.1-1.3). Given that the ability for sucrose to offset the bees aversion to alkaloids has previously been shown to be identical at 0.3 M and 0.6 M for nicotine, and 0.7 M and 1.0 M for caffeine, suggests that the concentrations of sucrose used in this thesis were simply within this ceiling effect range. i.e. if lower concentrations had been compared, e.g. 0.25 M and 0.5 M, a difference may have been observed. In order to examine whether taste is a factor in the bee's choice for solutions containing nicotine and caffeine, future experiments could focus on comparing the bee's responses to these alkaloids in lower (e.g. 0.25 M and 0.5 M) sucrose concentrations. Alternatively, as discussed previously (see 3.4.4), given that increasing the sucrose concentration that an alkaloid is administered in results in a decrease in the total volume consumed, and therefore the dose of the alkaloid consumed, future experiments may look to instead offer bees a choice in lower sucrose concentration, however, mask the taste of the alkaloids using a sweet non-nutritious sweetener such as saccharin (Burke and Waddell, 2011).

6.5. Do honeybees and bumblebees display addictive behaviour to nicotine and caffeine in line with the DSM-V/ICD10 criteria?

The Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 2013) and the World Health Organizations International Classification of Diseases (ICD10) serve as the two main diagnostic criteria in clinically defining substance abuse in humans (Hasin et al. 2013; Søvik et al. 2013). In order to be diagnosed as displaying addiction under the DSM-V and ICD10 criteria, three or more psychological or physiological symptoms need to be present concurrently. Psychological symptoms include sustained drug use despite adverse consequences, lack of control, an extensive amount of time devoted to drug acquisition, and compulsion. Whereas physiological symptoms include tolerance and withdrawal symptoms (detailed in table 1.2).

Although the work conducted in this thesis has identified preferential consumption of both nicotine and caffeine in honeybees and bumblebees, preferential consumption of drugs of abuse is not in and of itself indicative of addiction. Although the devaluation of higher molarity sucrose solutions observed in the bumblebee following three days pre-exposure to nicotine could perhaps be inferred as continued use despite adverse consequences, it is clear that insufficient criteria have been established in line with the DSM-V/ICD10 in order to classify bumblebees and honeybees as displaying an addicted state.

6.6 The evolutionary history of bees and the ecological function of secondary metabolites: Have honeybees and bumblebees adapted to consume nicotine and caffeine?

Mammalian models of addiction rely on the assumption that the animals that they use are unlikely to have any specific evolutionary adaptations that make them more or less susceptible to the effects of drugs than humans. If test animals are highly sensitive or highly resistant to the effects of a drug, then this confounds their ability to accurately model the human condition. The evolutionary history of insect pollinators is disparate from that of mammals due to their distinct ecological niche, and therefore insects have experienced markedly different selection pressures over time. Unlike rodents, insects may have encountered caffeine and nicotine throughout their evolutionary history due to the presence of these alkaloids within floral nectar and pollen. Therefore, if honeybees and bumblebees

show specific evolutionary adaptations to either alkaloid, this could confound their use as models with which to study addiction.

Insect pollinators that have been shown to tolerate toxic secondary metabolites found within floral nectar and pollen can, broadly speaking, be placed into two categories: specialist pollinators that have co-evolved to forage almost exclusively on a particular plant or generalist pollinators that have evolved alongside the plant and as such have been exposed to specific secondary metabolites over millennia (Wink et al. 2018). For example, the North American miner bee, *Andrena astragali*, is a narrow specialist, collecting pollen solely from two species of the neurotoxic alkaloid-producing genus *Toxicoscordion*, with no ill effects (Cane, 2018). In contrast, bumblebees and honeybees, both generalist pollinators, are known to avoid *Toxicoscordio* spp. (Cane, 2018), where consumption of their alkaloids results in their rapid death (Vansell and Watkins, 1933; Hitchcock, 1959). In this instance, the miner bee's specialism eliminates foraging competition from generalist bees, thus securing the plant's rewards for itself, whereas the generalist bee's lack of specialism causes them to lose the rewards of a single plant genus. Despite this, their ability to forage on multiple plants means they can simply choose to forage elsewhere without the need to develop specific and often energetically costly adaptations to tolerate toxic nectar (Du Rand et al. 2015).

An example of generalist pollinator adaptation can be seen in the case of *Rhododendron ponticum*, an invasive plant introduced to the British Isles from the Iberian Peninsula in the 18th century (Cross, 1975). *R. ponticum* is known to contain high concentrations of diterpenes known as grayanotoxins within its floral rewards (Tiedeken et al. 2015), and the nectar of this plant is known to be highly toxic to the native British bee, *Apis mellifera mellifera* (Tiedeken et al. 2015). Whereas bees native to the Iberian Peninsula: *Apis mellifera caucasia,* and *Apis mellifera anatolica,* readily forage on this plant with no ill effects (Sillici et al. 2008). This indicates that bees that have foraged on plants that produce toxic secondary metabolites within their floral rewards across their evolutionary history can become resistant to the effects of these compounds, whereas bees that are naïve to these compounds are more likely to be susceptible to their toxic effects. Note that in both examples of pollinator adaptations, the adaptation renders the insect more resistant to the effects of secondary metabolites, not more sensitive. Indeed, no studies to

date have identified that bees, other insects, or indeed mammals have specifically evolved to become more sensitive to the effects of secondary metabolites. Although there are instances where secondary metabolites can be phagostimulatory to some insects, i.e. the insect prefers the taste of these compounds, this appears constrained to specialist herbivores and secondary metabolites that aid in host plant recognition (Stevenson et al. 2017; Pentzold et al. 2017). Indeed, secondary metabolites are toxins, and detoxification is metabolically costly (Du Rand et al. 2015); therefore, the likelihood of selection pressure being applied to insects to preferentially consume these compounds is low. Therefore, if the bee species used in this thesis have evolved to adapt to either nicotine or caffeine, greater tolerance (i.e. resistance to these compounds toxic effects) rather than sensitivity would be expected. In addition, if these bees have evolved to tolerate nicotine or caffeine, it is expected that there is evidence that the bee species used in this thesis either display some form of specialism to forage on plants that produce these secondary metabolites within their floral nectar or, that they have co-evolved with plants that contain these compounds across their extended evolutionary history.

The bees used in these experiments: *Bombus terrestris audax* and *Apis mellifera* (var. *carnica* and *buckfast*), are all European bee species (Goulson, 2010; Ilvasov and Kwon, 2019). *Nicotiana* spp. are native to the Americas, Africa, and Australia (Clarkson et al. 2004); however, they were introduced to Europe in the 16th century (Vetulani, 2001). Although not grown for commercial tobacco production, several ornamental varieties of *Nicotiana* are commonly grown in European gardens (Cooper and Johnson, 1984). *Nicotiana* spp. have distinctly elongated corollas (McCarthy et al. 2016), and as such, bees are likely to struggle to access the nectaries of these plants (Cariveau et al. 2016). Indeed, *Nicotiana* spp. are typically pollinated by insects with long proboscises, such as the tobacco hawk moth (Kessler et al. 2010), or birds with elongated beaks, such as hummingbirds (Kessler et al. 2010), or sunbirds (Marlin et al. 2016). However, some bee species have been shown to be legitimate pollinators of *Nicotiana* spp. albeit highly infrequently (Schueller, 2004; Ollerton et al. 2012. Note: bee species not provided). Indeed, bees, including bumblebees, honeybees, and carpenter bees, instead function as nectar robbers of *Nicotiana* spp. where they pierce the corolla to access the nectar without coming into contact with the plant's sexual organs (Ollerton et al. 2012). Although bees may also encounter nicotine in other members of the *Solanaceae*, such as tomatoes or peppers, the

concentrations of nicotine within these tissues are many orders of magnitude lower than that found in *Nicotiana* spp. (in the order of 2-7 µg/kg of plant tissue for tomatoes, and 3.9 g/kg in *Nicotiana tabacum*) (Siegmund et al. 1999; Martinez et al. 2019). This coincides with the low expression of nicotine biosynthetic genes in these plants (Xu et al. 2017). No studies to date have assessed whether nicotine is present within the floral nectar and pollen of these members of the *Solanaceae*; however, given the low rate of nicotine biosynthesis in these species, if nicotine is present, it is likely to be at such a low concentration as to have negligible pharmacological effects. Therefore, the European bee species used in these studies have been spatially separated from *Nicotiana* spp. for much of their evolutionary history (i.e. up until the 16th century). Although bees may have encountered nicotine in their recent evolutionary history by nectar-robbing ornamental *Nicotiana* spp. present across Europe, the generalist foraging behaviour of these bee species, and the likely infrequency with which they encounter plants grown purely for ornamental purposes amongst a plethora of alternative flowers make specific evolutionary adaptations to nicotine in these bee species highly unlikely, i.e. there is likely very weak selection pressure for the bee species used in this thesis to develop resistance to nicotine; therefore, it is not assumed that the bee species used in this thesis have any evolutionary adaptations that would confound their use in modelling nicotine addiction.

Honeybees and bumblebees encounter nicotine in the range of ~0.5-30 µM in the nectar (Singaravelan et al. 2005; Tadmor-Melamed et al., 2004; Kessler et al., 2010), and up to 140 µM in the pollen of *Nicotiana* spp. Although both honeybees and bumblebees exhibit a preference for ecologically relevant concentrations of nicotine in a concentration and schedule dependent manner (detailed in chapter 3.0 and 4.0), it is unlikely that they encounter nicotine within the field frequently enough (e.g. chronically for a period of 3 days) for ecologically relevant concentrations of nicotine to have a strong effect on bee behaviour in the field.

Caffeine is present within the floral nectar and pollen of *Coffea*, *Citrus*, and *Tilia* spp. (Wright et al. 2013; Koch and Stevenson, 2017), and all three genera are known to be regularly pollinated by honeybees and bumblebees (Monroy et al. 2015; Abrol, 2015; Koch and Stevenson, 2017). *Coffea* spp. are indigenous to Africa (Razafinarivo et al. 2013) and typically require tropical conditions and high altitudes to grow successfully (Davis et al.

2012); as such, *Coffea* is not typically grown in the UK. *Citrus* spp. are native to Asia and Australia (Pfeil and Crisp, 2008) and have been cultivated in Europe (Mediterranean) since the 10th century (Forsyth and Damian, 2003; Rámon-Laca, 2003). Finally, *Tilia* spp. are native to Asia; however, they were introduced to Europe in the 16th century (Koch and Stevenson, 2017), where they are now one of the most common genera of trees grown in European towns and cities (Pauleit et al. 2002). The concentration of caffeine in *Tilia* is ~260 μM and is found in the pollen of the plant (Unpublished data, Kew). Interestingly, there are numerous reports of dead bumblebees, and to a lesser extent, honeybees, being found under flowering *Tilia* trees across Europe (Koch and Stevenson, 2017). Given that caffeine is known to manipulate bee behaviour to increase foraging fidelity (discussed in 1.2 and chapter 5.0), one possible explanation that could account for the mass deaths in bees is that the caffeine present within the floral rewards of *Tilia* are causing bees to return to the plant long after the plant has ceased nectar secretion, ultimately resulting in the starvation and death of bees that forage on these plants (discussed extensively in Koch and Stevenson, 2017). Assuming that this hypothesis is correct, it is plausible that reasonable selection pressure may be present on European honeybees and bumblebees to become resistant to the pharmacological effects of this alkaloid. That is, if bees were resistant to the pharmacological effects of caffeine, bees would learn to forage on alternate resources once the plant has ceased nectar secretion; this would ensure forager survival and thus the overall fitness of their colonies. However, given that mass deaths have been reported since the time of the plant's introduction to the British Isles, and deaths are still occurring to this day (Koch and Stevenson, 2017), it appears that neither honeybees or bumblebees have developed resistance to the pharmacological effects of caffeine over the last 400 years. Indeed, the results detailed in chapter 5.0 show that even though honeybees avoid low concentrations of caffeine initially, they did not avoid higher concentrations of the alkaloid that are also within the range of caffeine concentrations found in *Tilia* spp. In addition, the honeybee's avoidance of low concentrations of caffeine instead turned to preference once honeybees had been pre-exposed to caffeine for three days chronically. Although an initial aversion to low concentrations of caffeine may confer an advantage to the honeybee, i.e. honeybees that encounter low concentrations of caffeine in *Tilia* spp. may be more prone to avoid these plants, the fact that they do not avoid a range of other ecologically relevant caffeine concentrations suggests that any benefit this may provide them is likely only weak. In addition, bumblebees did not show aversion to caffeine in any of the schedules used,

including caffeine concentrations greater than that observed in *Tilia* spp. Instead, bumblebees displayed a preference for caffeine following three days pre-exposure to the compound. This suggests that honeybees and bumblebees are susceptible to the pharmacological effects of caffeine, and therefore may serve as models with which to study caffeine addiction. Despite this, future studies should be cautious. If evidence arises to suggest that either bee species possesses neural or molecular adaptations due to encountering caffeine within their recent evolutionary history, then the use of these bee species as viable models of caffeine addiction should be reconsidered.

Unfortunately, caffeine concentrations of ~260 μM (i.e. the concentrations found within *Tilia* spp.) were not tested in this study. Given the mass deaths observed in bees that forage on *Tilia* spp., and the preference observed in bumblebees pre-exposed to intermediate concentrations of caffeine (100 μM), it may be beneficial to repeat this study using the concentrations of caffeine found within *Tilia* spp. to assess whether chronic pre-exposure to this concentration of caffeine affects the bees subsequent choice behaviour.

6.7. Research limitations

In addition to the confound of age on preferential consumption of drugs of abuse (discussed above), this study was also limited by the methodology employed for the unexposed treatment groups. Unexposed honeybees were shown to consistently consume a greater total volume of solution during the 24 h choice period than pre-exposed bees, and similar results were observed in some, but not all, bumblebee studies. I hypothesise that the increased consumption in unexposed bees is likely explained by the stress and temporary starvation that occurs during bee capture and restraint, as similar increases in consumption are observed in pre-exposed honeybees over the first day of the pre-exposure period. This resulted in unexposed bees consuming a greater total dose of caffeine or nicotine over the 24 h choice period, in comparison to pre-exposed bees, and thus hinders the direct comparison of unexposed and pre-exposed treatment groups in many studies. In order to avoid this, future studies are recommended to maintain unexposed bees on sucrose alone for a period of three days to equilibrate the bee's nutritional requirements over the 24 h choice periods between treatment groups, thus making the dose consumed directly comparable.

Given that it is possible that bees are experiencing stress during capture and restraint, stress may also be a confound in the experiments conducted in this thesis. In rodents, both acute and chronic stress, such as tail pinches or social isolation, are known to increase the stress hormone corticotropin releasing factor and facilitate drug-seeking behaviour (Piazza and LeMoal, 1998; Samarghandian et al. 2003; Backström and Winberg, 2013; Zorrilla et al. 2014). Indeed, in *Drosophila*, stressful situations such as mating rivalry and social isolation have been shown to lead to increases in the invertebrate ortholog of corticotropin releasing factor in the brains of flies (Mohammad et al. 2016; Mohorianu et al. 2017). Given that both honeybees and bumblebees are eusocial insects, the removal of these animals from conspecifics and the stress that is likely endured through the restraint procedure may influence the bee's willingness to consume addictive substances. In addition, although honeybees were housed in cohorts of 20 bees, bumblebees were housed individually, which may suggest that bumblebees are experiencing more stress during these assays than honeybees. It is important to note that although the possible introduction of stressors may influence bee behaviour in these studies, stressors are also a caveat in rodent studies. For instance, rodents, like bees, are social animals (Wilson and Koenig, 2014), and addiction studies typically result in the social isolation of test animals (Rappeneau and Bérod, 2017). In addition, rodents often undergo invasive surgeries to prepare them for addiction studies, such as cannula implantation, which is known to be highly stressful to these animals (Spanagel, 2017). Therefore, the caveat of stress introduction is similar in both insect and mammalian studies. Future studies may control for these stressors by either looking at addiction behaviour at the colony level (e.g. with whole colonies of bumblebees in free-flight settings which would avoid individual insect capture and restraint), or perhaps by looking to enrich the caged environment, such as by providing comb which carries the pheromones from the insect's colony of origin and more closely resembles the bees natural environment. (Williams et al. 2015). In addition, although bees were placed on ice temporarily during capture and restraint of the bees, which allows for rapid placement of bees within test cages, it is possible to introduce bees to their test cages without the freezing procedure, which may further minimise the stress endured.

6.8. Future studies

In addition to the future studies detailed above. Further studies that would continue the findings reported in this thesis include:

- Assessment of the ontogeny of choice behaviour: Preferences have been identified for nicotine and caffeine in honeybees and bumblebees when two-way choice assays are conducted over short periods of time in free-flight settings (1-3 h); however, they are not observed over 24 h two-way choice in a caged setting within the laboratory. This suggests that preferential consumption of these alkaloids varies depending on either the type of assay conducted (free-flight versus caged assays) or the duration of alkaloid access. In order to establish the ontogeny of alkaloid preference over time, future experiments may look to examine preferential consumption of these alkaloids over a narrower range of time points. As discussed earlier, a caged capacitance-based feeding system has recently been developed in the Wright laboratory (Unpublished data), which allows for high-resolution (in the order of ms) quantification of the bees feeding behaviour throughout a 24 h period. This methodology would identify if caged bees show an initial preference for either alkaloid in a caged setting, as is apparent in free-flight assays, or whether the bee is largely indifferent to either alkaloid throughout the 24 h two-way choice periods. This would therefore elucidate whether it is the type of assay conducted that accounts for this difference in preferential consumption or the duration of alkaloid exposure.
- Direct assessment of tolerance and withdrawal symptoms following nicotine pre-exposure in both bee species - Tolerance is typically assessed as an attenuation of the enhanced locomotor response following pre-exposure to a drug (see section 1.2.3). Numerous methodologies exist that allow for the assessment of insect locomotion (Mohammad et al. 2016) and can be used to infer whether tolerance to either alkaloid occurs in the bee. Withdrawal symptoms are classified as somatic, affective, or cognitive (McLaughlin et al. 2015). Somatic symptomology can be assessed by examining whether bees exhibit ‘malaise-like’ behaviours during alkaloid abstinence. Malaise has been characterised in the bee and is observed behaviourally as an inability to perform the righting reflex, abdomen dragging, and curling up (Hurst et al. 2014). In addition, behavioural paradigms exist to examine ‘affective-like’ states in insects. For instance, anxiety-like behaviours can be assessed by examining how an insect

interacts with an open field maze, identical to how anxiety is measured in rodents (Mohammad et al. 2016). Also, 'pessimism-like' states have been shown in the honeybee, whereby ambiguous stimuli are predicted as punishment in olfactory conditioning of the proboscis extension reflex (Bateson et al. 2011). Finally, cognitive deficits, such as difficulties in learning and forming memories, can be assessed by training and testing bees in olfactory conditioning of the proboscis extension reflex (Giurfa and Sandoz, 2012).

- Direct assessment of the psychological symptoms detailed in the DSM-V/ICD10 criteria - Although identification of physiological symptoms such as tolerance and withdrawal aid in identifying the underlying motivation to consume drugs of abuse, tolerance, and withdrawal symptomology alone are insufficient to be classified as an addict under the DSM-V/ICD-10 criteria, and psychological symptoms must be present concurrently (See table 2.1). Therefore, future studies are required to assess whether psychological symptoms are present in the bee following pre-exposure to caffeine and nicotine. For instance, "a great deal of time is spent in activities necessary to obtain the substance" can be assessed in free-flight paradigms, whereby artificial flowers that contain nicotine or caffeine are made more difficult to access in comparison to flowers that contain sucrose alone (Giray et al. 2015). In addition, "continued use despite harm" has been identified in *Drosophila* in response to ethanol, whereby flies are willing to overcome negative stimuli (electric shocks or a bitter-tasting compound) in order to obtain ethanol or to gain access to a cue previously associated with the substance (Kaun et al. 2012). Similar paradigms could be adapted for the bee to assess psychological symptoms in line with the DSM-V/ICD10 criteria.
- Intermittent paradigms have only been conducted with honeybees, and it is not clear what effect periods of abstinence will have on bumblebee choice behaviour. Therefore, future studies may look to assess how intermittent schedules of administration affect the bumblebee's choice behaviour for nicotine and caffeine.
- It is not clear what molecular or neurological changes occur throughout the pre-exposure period to account for the bee's preferential consumption of nicotine or caffeine. Future studies may therefore look to examine whether changes in gene expression may account for this. For example, changes in corticotropin releasing factor, nAChR, and adenosine receptor expression may aid in identifying the neurological substrates responsible for generating this behaviour. Assessment of these changes, in combination with pharmacological or gene knock-down technology, would serve as a

powerful means to identify the molecular substrates responsible for the behaviour observed in this thesis.

- Previous studies have identified the rate of clearance (i.e. detoxification) of high concentrations of nicotine in the honeybee (Du Rand et al. 2017); however, the concentrations used in this thesis have not yet been examined. In addition, the clearance rate of nicotine in the bumblebee, and caffeine in both bee species, has not yet been assessed. The pharmacokinetics of a drug are important in mediating its addictive potential (Allain et al. 2015). Therefore, future studies may look to examine how nicotine haemolymph concentrations change across the schedules of administration used in this thesis. This may aid in identifying why the intermittent 12 (I12), and intermittent 48 schedules (I48), generate preferences for different concentrations of nicotine in the honeybee. In addition, it will help guide future withdrawal studies by identifying the time point when withdrawal symptomologies may occur.

6.9 Conclusion - Are honeybees and bumblebees useful models with which to study caffeine and nicotine addiction?

The research conducted in this thesis provides only weak evidence that honeybees and bumblebees may serve as viable models with which to model caffeine and nicotine addiction. Indeed, the strongest evidence presented is that of honeybees that have undergone either the intermittent 12 or intermittent 48 exposure schedules of administration for nicotine, which is suggestive of withdrawal. Although preferences are observed for both nicotine and caffeine in both species, depending on the schedule and administration used, these responses do not follow the expected biphasic dose-response curve. Further studies using narrower concentration ranges of both alkaloids are needed to determine if biphasic responses to these alkaloids are present in bees, as would be expected of an addictive compound.

Collectively, the evidence collected in this thesis is insufficient to validate the use of honeybees and bumblebees as viable models for nicotine and caffeine addiction, and more behavioural research is required to ascertain whether they show sufficient criteria in line with the DSM-V and ICD10 guidelines for addiction. In addition, studies that assess the neurological and molecular changes that occur following caffeine and nicotine consumption, as well as pharmacokinetic studies assessing the rate of alkaloid degradation, would be beneficial in validating the use of these insects to study addiction. This would help ascertain

whether bees undergo the same underlying molecular and neurological changes that occur in mammals during their exposure to each alkaloid, a critical requirement if they are to model the human condition. Despite the fact that numerous studies are clearly required in order to ascertain whether bees may serve as viable models for addiction, honeybees and bumblebees are exceptional models with which to study learning and memory, as well as behaviour. In addition, their simplified neural circuitry would be beneficial to future addiction studies if their viability as a model is confirmed. Indeed, even if future research does not provide ample evidence that bees can serve as models with which to study the human condition, research into whether these compounds exert addictive-like properties on bees would be beneficial to ecological studies and would greatly aid in understanding plant-pollinator interactions.

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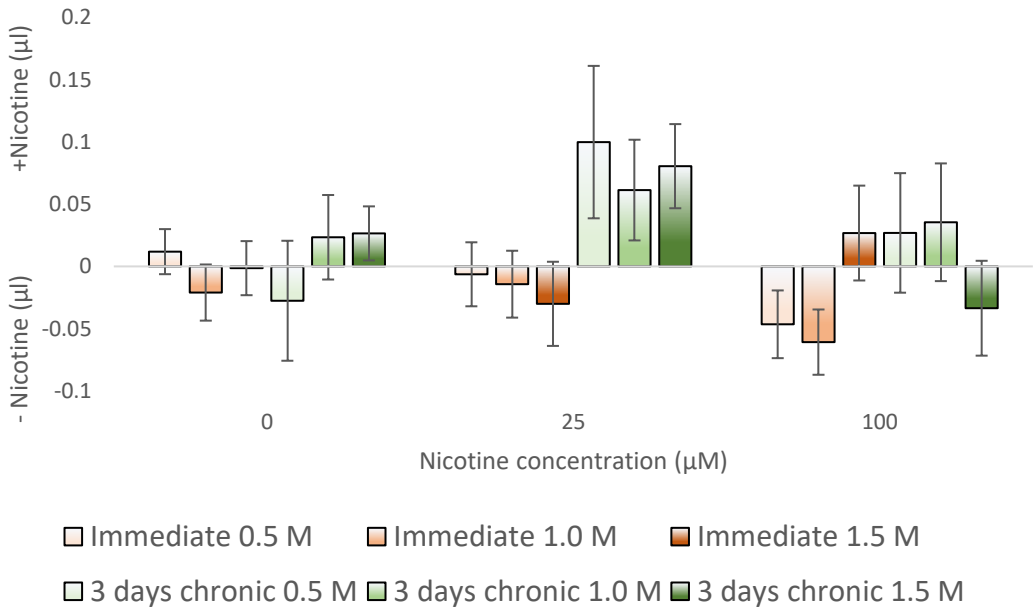
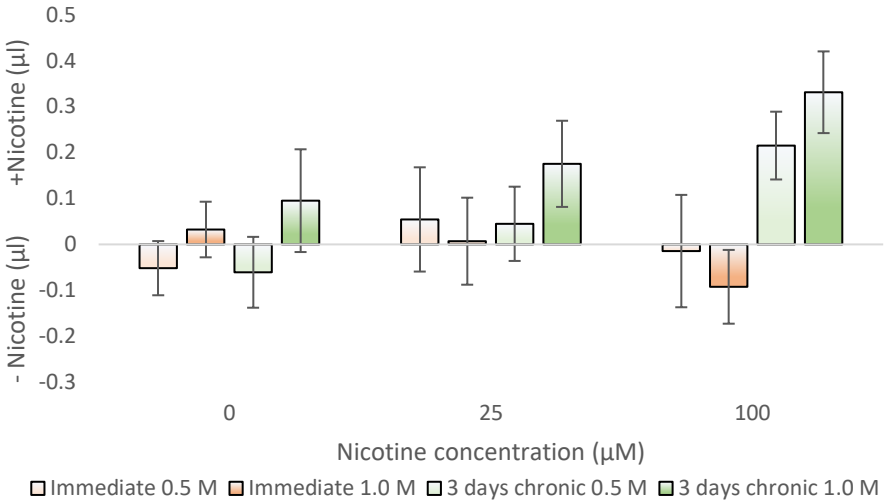
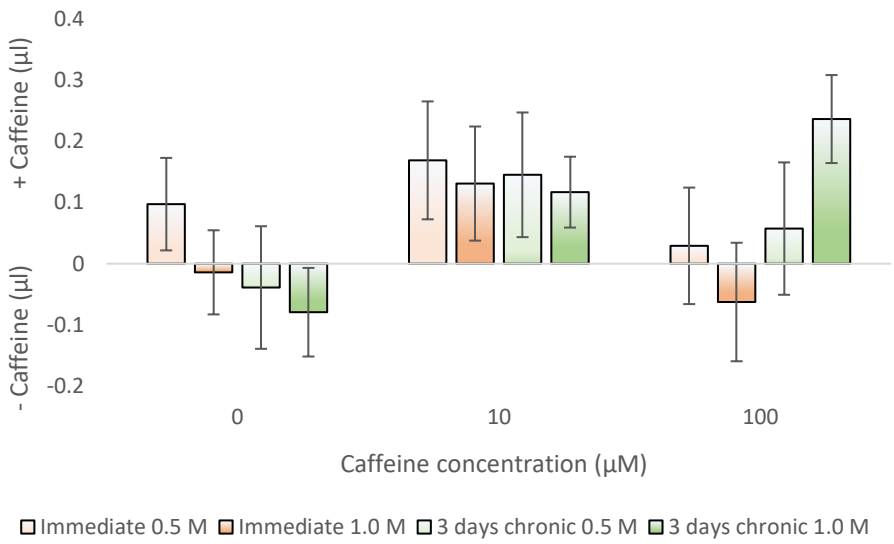
Appendix



Supplementary figure 1.1 | Increasing the sucrose molarity nicotine is administered in does not affect the total preference for the compound in the honeybee. Combined immediate 24 h two-way choice and 3 day pre-exposure indexes for nicotine preference in the honeybee. Preferences for nicotine vary as a function of the exposure period (GzLM main effect of Exposure: $\chi^2_{(1)} = 7.66$, $p = 0.006$), but not as a function of treatment (GzLM main effect of treatment: $\chi^2_{(8)} = 4.84$, $p = 0.775$. GzLM, Exposure x treatment: $\chi^2_{(8)} = 10.5$, $p = 0.229$). Data represent the mean difference (±s.e.m.) in the amount consumed (µl) per bee over 24 h, controlled for by evaporation. Positive values indicate a preference for nicotine and negative avoidance of nicotine. Sample sizes detailed in 3.3.



Supplementary figure 1.2 | Increasing the sucrose molarity nicotine is administered in does not affect the total preference for the compound in the bumblebee. Combined immediate 24 h two-way choice and 3 day pre-exposure indexes for nicotine preference in the honeybee. Preferences for nicotine vary as a function of the exposure period (GzLM main effect of exposure: $\chi^2_{(1)} = 6.88$, $p = 0.009$), but not as a function of treatment (GzLM main effect of treatment: $\chi^2_{(5)} = 4.85$, $p = 0.435$. GzLM, Exposure x treatment: $\chi^2_{(5)} = 8.35$, $p = 0.138$). Data represent the mean difference (±s.e.m.) in the amount consumed (µl) per bee over 24 h, controlled for by evaporation. Positive values indicate a preference for nicotine and negative avoidance of nicotine. Sample sizes detailed in 4.3.



Supplementary figure 1.3 | Increasing the sucrose molarity caffeine is administered in does not affect the total preference for the compound in the bumblebee Combined immediate 24 h two-way choice and 3 day pre-exposure indexes for caffeine preference in the bumblebee. Preferences for caffeine do not vary as a function of the exposure period (GLM main effect of exposure: $F_{(1, 277)} = 0.084$, $\eta_p^2 = <0.001$, $p = 0.772$), or concentration (GLM main effect of exposure: $F_{(5, 277)} = 1.381$, $\eta_p^2 = 0.024$, $p = 0.231$). Nor was there an interaction effect (GLM exposure concentration: $F_{(5, 277)} = 1.523$, $\eta_p^2 = 0.027$, $p = 0.183$). Data represent the mean difference (±s.e.m.) in the amount consumed (µl) per bee over 24 h, controlled for by evaporation. Positive values indicate a preference for nicotine and negative avoidance of nicotine. Sample sizes detailed in 5.3.