The effects of cholinergic drugs in nectar on bee feeding behaviour



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Declaration

This thesis is the result of my own work. Where people contributed to the collection of data this has been declared in the acknowledgements. This work has not been submitted for any other degree or professional qualification.

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Abstract

Flowering plants often recruit bees as pollen vectors by attracting them with floral nectar. Plants offering more valuable nectars are more successful at conditioning pollinators to visit them, which promotes pollination and outcrossing. Intriguingly, certain flowering plants such as *Nicotiana* sp. produce nectar spiked with secondary metabolites that can interact with receptors in the insect nervous system.

Nicotine and related nectar drugs mimic acetylcholine (ACh), a neurotransmitter critical for associative learning in insects. ACh receptors are also expressed in dopaminergic neurons involved in the formation of reward memories. These compounds may therefore have the potential to pharmacologically enhance learning of nectar rewards when experienced by pollinators during foraging and bias their feeding decisions.

Compounds that can usurp the reward-encoding activity of dopaminergic neurons can also drive unconstrained drug approach behaviours. Recurrent exposure to nicotine can produce compensatory changes in the number, sensitivity and composition of receptors expressed in the brain. This can alter its chemical homeostasis and potentially lead to drug dependence and addiction. Whether this group of drugs can make nectar addictive to bees is unknown and largely unexplored.

In this thesis, I use a combination of behavioural assays to investigate the cognitive and potentially addictive effects of dietary exposure to nicotine and other chemically related nectar drugs in bees.

In chapter 2, I present two experiments that test how field-relevant nicotine-laced food influences feeding preference and reward learning in honeybees. In a binary choice assay I found that nicotine exposure tended to bias choice behaviour, but bees only developed robust nicotine preferences when in withdrawal. I then used an olfactory conditioning assay to examine directly whether nicotine in food impacted reward learning and/or memory, and whether this depended on previous exposure to nicotine. The drug had equivocal effects on naive bees but those that had been pre-exposed to it showed patterns of responding to either reward type that were consistent with the addiction hypothesis.

To assay perceived distinctions in reward value that may be slight, in flux with an animal's homeostatic state, and dependent on individual variation; I developed a computer-based system for tracking binary feeding choice behaviour in individual bumblebees at fine behavioural scales. In chapter 3 I present data from a pilot study using quinine, verifying that this assay is sensitive to the detection of aversive feeding in bees and can reveal information about the dynamics of feeding decisions through time.

Chapter 4 details two experiments that tested for withdrawal-induced drug seeking, a hallmark addictive behaviour associated with drug-dependence, in response to a selection of putatively addictive nectar compounds. In the first, I validated in bumblebees that nicotine intake is increased with pre-exposure and withdrawal and revealed the temporal dynamics of nicotine preference: naive and pre-exposed bees showed an initial burst of nicotine-seeking that was likely driven by state-dependent post-ingestive reinforcement. In a second experiment, I screened a selection of other nectar-relevant cholinergic drugs for their potential to induce similar behavioural changes through pre-exposure and withdrawal. Arecoline, another nAChR agonist, produced preferential feeding and nicotine-like patterns of responding. Responses to thiamethoxam, scopolamine, and caffeine were more ambiguous with respect to the addiction hypothesis. These effects are likely to be highly dependent on dose, mechanism of action, and molecular structure.

Chapter 1 | Introduction

Evolutionary dynamics of nectar chemistry

Flowers are multisensory advertisements produced by most plants to lure pollinators into contact with their reproductive structures and vector gametes between receptive conspecifics. Pollinators gather their nutrients from plants in exchange. These relationships are mutualistic, which is to say transactional: they harbour a degree of tension that exists between the conflicting interests of each party and are subject to marketplace conditions.

Bees are generally perceived to be the preeminent group of pollinators for various reasons including their effectiveness (Sárospataki 2020), specialisation (Ollerton 1999), and abundance (Willmer et al. 2017). Nearly all bees depend on pollen and floral nectar for their nutrition (Michener 2007). Pollen is the male gametophyte of plants. To bees, it is a source of protein, starch, sterols, and lipids (Furse et al. 2023, Roulston et al. 2000). Nectar, on the other hand, serves no direct reproductive role but is produced specifically to attract and reward pollinators.

Nectar is a solution of sucrose, glucose, and/or fructose, and occasionally other sugars; the ratio of which seems to correspond with the species of pollinators it attracts (Baker and Baker, 1990; Wright lab, unpublished data). For bees, nectar is their main source of energy (Nicolson 2022). We can consider it the currency of the plant-pollinator economy. Plants often contend with one another for these services (Jones et al. 2012), and are consequently under selection pressure to provide bees with competitive nectar that encodes salient memories of food reward in the brain to incentivise future visits to conspecific plants (Parachnowitsch et al. 2018). The brains and behaviour of bees are in turn under selection to exploit these resources efficiently: they have refined mechanisms for assessing the quality, quantity, and probability of re-encountering different nectars, and learning and remembering where to find them (Chittka 2017).

Many mutualisms have their origins in parasitism (Johnson et al. 2021, Weiblen & Treiber 2015). This could potentially also be true of plant-pollinator relationships, which might have evolved from plants co-opting antagonists that consumed their reproductive tissues and structures (Crepet et al. 1991, Labandeira et al. 2002, Weiblen & Treiber 2015). The persistence of stable cooperation between plant and pollinator has not been difficult to account for in theory (Axelrod & Hamilton 1981, Bull & Rice 1991, Herre et al. 1999, Briggs et al. 2019, Ferriere et al. 2002) but it nonetheless happens that flowers can evolve to deceive pollinators in various ways like feigning rewards (Schiestl 2005) and mimicking more rewarding flowers (Nilsson 1983, Anderson & Johnson 2006, Johnson 2000, Peakall 2023) – the deception of orchids can be particularly devious (Gaskett et al. 2008, Brodmann et al. 2009) – and pollinators can evolve to remove nectar without pollinating plants (van der Kooi et al. 2021, Irwin et al. 2010, Zhang et al. 2021). The threat that these relationships will degenerate back into a parasitic state is arguably never far away (Smithson 2009, Leigh 2010).

Floral signals can therefore be deceptive (van der Kooi et al. 2023, Sun et al. 2018) and serve to manipulate decisions made by pollinators about whether to visit a flower. If a nectar payout is received, it is experienced only once visitation is already complete. Nectar isn't typically thought of as a floral signal despite carrying information in its energetic value that is used by pollinators to inform their future foraging decisions. We might therefore expect the opportunity for manipulation to cease once this transaction is completed and the nectar is ingested. But what if the signalling game between plant and pollinator could persist post-ingestively?

The evolutionary paradox of nectar SMs

Plants produce an incredible diversity of chemical compounds that are not directly required for their growth or reproduction but serve evolved ecological roles, particularly in defence against antagonists such as insect pests, fungi, and microbes (Pichersky & Gang 2000, Bennett & Wallsgrove 1994). Many of these secondary metabolites (SMs) have insecticidal properties and accumulate in the vegetative tissues, with well-explored functions in defense against herbivory. However, SMs that are the same or chemically similar may be present in the floral nectar. Proximally, the identity of these compounds can be explained by the fact that the chemical weaponry of an individual plant tends to be restricted to a small set of biochemical pathways (Stevenson 2020). How-

ever, defence theory (McCall & Fordyce 2010) and ecological costs (Adler 2000) suggest that these compounds probably ought only to be found in the critical tissues, and the reasons for the observed rate at which these toxins occur in nectar and their role in interactions with pollinators are not self-evident nor well understood (Stevenson 2020). Nectar is synthesised specifically as a reward to bees, with nutritional profiles tailored to their needs (Baker & Baker 1975, Parkinson et al. 2025); and its apparent contamination with toxins has been regarded as an evolutionary paradox.

Floral nectar is produced in glands known as nectaries, which import, synthesise, and enrich the contents of the phloem and/or xylem with other nutrients (Heil 2011). Toxic nectar could conceivably be a maladaptive symptom of a pleiotropic trade-off that exists due to an inability to decouple SM expression in flowers and foliage. Little is known about the mechanisms of SM synthesis in nectar (Heil 2011), but if SMs are transported through the plant's vascular system rather than being actively synthesised by the nectaries it may be that they leak passively into nectar in a way that evolutionarily has been difficult to constrain because of antagonistic selection processes. The observed pattern of toxin expression between tissues and groups of plants indicates that this could be the case, at least in certain plant species: concentrations of defensive compounds across *Nicotiana* tissues tend to be lower in outcrossing species, for example (Adler et al. 2012) which perhaps implies the presence of some pollinator-mediated selection to lower their expression. But there are also many instances when the chemical blends of nectar indicate that this explanation is insufficient (Cook et al. 2013, Prys-Jones & Willmer 1992, Egan et al. 2016, Wright et al. 2013). Several recent reviews have synthesised the evidence for proposed hypotheses about the adaptive functions of toxic nectar (Adler 2000, Irwin et al. 2014, Stevenson et al. 2017, Barberis et al. 2023). It wouldn't be surprising if toxic nectar SMs served to mediate plant-pollinator interactions in diverse ways across different systems.

Rhoades & Bergdahl (1981) argued that nectar chemistry might encourage visitation by specialist pollinators because long-tongued bees appear to be more resistant to nectar alkaloids. In theory, toxic or distasteful nectar could therefore serve to fend off more generalist pollinators and improve the rates of conspecific pollen transfer, but empirical evidence for this is quite scant. Plants may also be able to repel nectar robbers in a similar way by exploiting the differential toxin sensitivities of pests and pollinators (Janzen 1977). This makes some potentially unjustified assumptions about the effects of nectar robbery on plant fitness (Castro et al. 2008, Morris 1996, Arizmendi et al. 1996, Ye et al. 2017, Varma & Sinu 2019, Navarro 2000) but it might be accurate in certain systems (Stephenson 1981, 1982, Euler & Baldwin 1996, Barlow et al. 2017, Kessler et al. 2008).

Another related hypothesis holds that distasteful alkaloids in nectar can help plants to economise on nectar costs while optimising the amount of floral visitation. In some cases, bitter tasting nectar might encourage pollinators to leave plants after sampling from only a few flowers, thus improving pollen export and hence the plant's success of outcrossing (Irwin & Adler 2008, Kessler & Baldwin 2007).

Otherwise, certain alkaloids may help to protect nectar nutrients against degradation by microbes (Adler & Wink 2001, Hagler & Buchmann 1993, Carter & Thornburg 2004) with which it is easily contaminated (Herrera et al. 2009, Fridman et al. 2012, Aizenberg-Gershtein et al. 2015). They may even benefit bees by warding off parasites (Richardson et al. 2015, Baracchi et al. 2015, Manson et al. 2010).

Intriguingly, certain nectar SMs exert their insecticidal action by interacting with receptors expressed in the insect nervous system (reviewed by Mustard (2020). This means that sublethal nectar-relevant concentrations of compounds that affect neurotransmission could potentially influence the mechanisms of learning and memory in pollinators and ultimately, in consequence, their foraging behaviour.

Neurophysiology of reward learning

Associative learning allows animals to learn to predict the onset of biologically important outcomes like food or danger from the occurrence of environmental stimuli. For bees, floral landscapes are complex and contain a variety of multisensory information that correlates with the presence of food rewards that vary in their perceived taste and nutritive qualities. Bees acquire and integrate these stimuli to form memories that can be stored and recalled to coordinate future approach or retreat behaviour as adaptive.

Classical appetitive conditioning typifies this process. It involves the presentation of a conditioned stimulus, such as an auditory cue, followed by the delivery of an unconditioned food stimulus (Pavlov 1927). The brain gathers and synthesises this pattern of sensory input to form an associative memory, and the conditioned stimulus acquires predictive utility. When bees contact a sugary solution with their antennae they reflexively extend their proboscis, but once an appetitive association is formed the conditioned stimulus alone can activate this response. Bees can learn to make

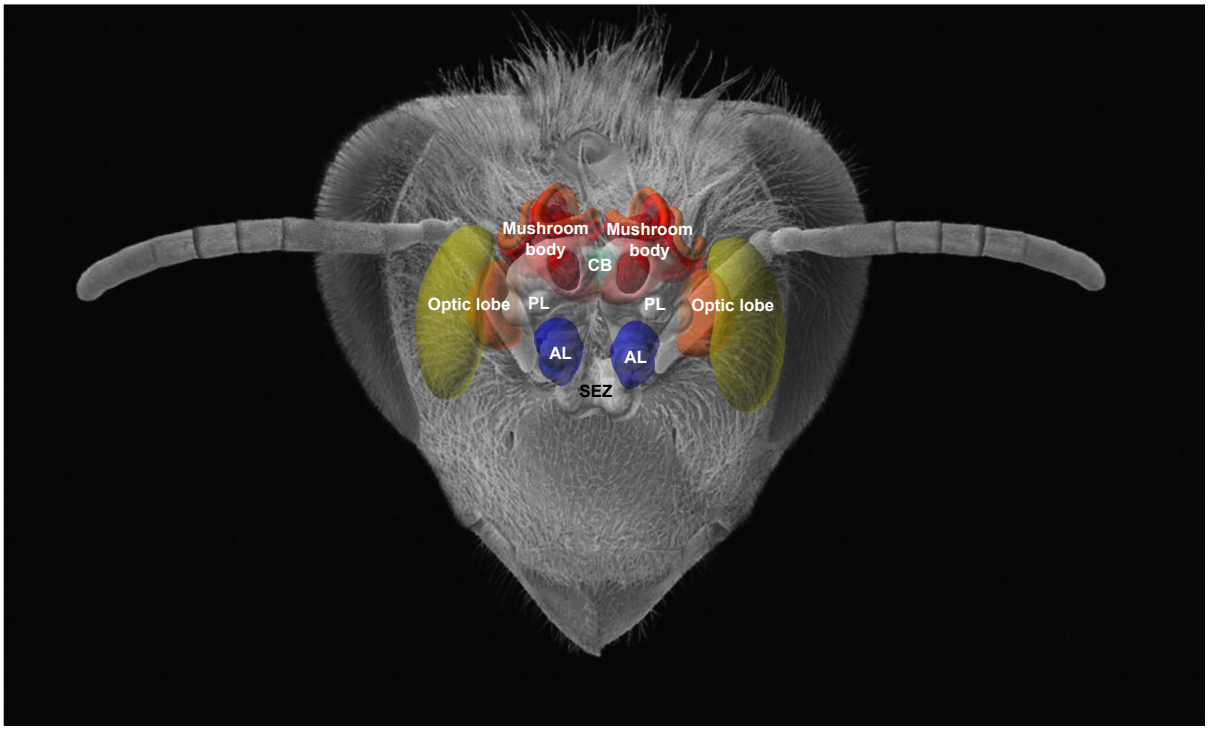


Figure 1 | **Labelled structures of the honeybee brain**. AL = antennal lobe, SEZ = sub-esophageal zone, PL = protocerebral lobe, CB = central body. Photo by Dan Winters; brain diagram from the Insect Brain Database.

use of cues in multiple sensory modalities, including specifically in an appetitive learning context (Gil-Guevara et al. 2022, Giurfa & Malun 2004); but their brains are specialised for doing so with odours because ecologically the experience of floral scents and nectar rewards are tightly coupled.

When a foraging bee encounters an odour, it is detected by olfactory receptors hosted in the membranes of olfactory receptor neurons (ORNs) in the antennae. The ORNs project signals up to the antennal lobe (AL; Fig. 1), where odour identity and concentration is reflected in a specific spatiotemporal pattern of firing in this population of excitatory and inhibitory neurons (Perez-Orive et al. 2002, Stopfer et al. 2003). The AL then relays this information via specific patterns of activity in projection neurons to the higher centres of the brain – notably the mushroom bodies (MBs), which are well-studied brain structures that are very important for learned behaviour in arthropods. In the MB, odour identity is re-encoded spatially according to which of a sparse subset of its constituent cells (Kenyon cells, KCs) are activated (Campbell et al. 2013, Lin et al. 2014, Barnstedt et al. 2016).

Meanwhile, tastants in nectar activate the bee's gustatory system. Gustatory receptor neurons (GRNs) in the antennae project signals to the dorsal lobe (Suzuki 1975, Haupt 2007), whereas GRNs in the its proboscis project up to what is now known as the subesophageal zone (SEZ). Some gustatory receptors are also expressed internally in the insect body, including the gut (Park & Kwon 2011) and the MB (Miyamoto et al. 2012), which may provide insects with an additional, more direct mechanism for monitoring their nutrient levels.

The SEZ is analogous in function to the AL in that it serves as a primary processing centre, but it receives taste input. It is more diffuse, however, and less well studied. The SEZ makes use of immediate taste and mechanosensory feedback from the proboscis to govern the motor output of feeding (Wright 2016), and projects post-ingestive signals via the 'taste tract' (subesophageal-calycal tract) to the MBs (Schröter & Menzel 2003, Yagi et al. 2016).

Olfactory and gustatory information thus converges in the mushroom body. When experienced simultaneously, the presynaptic terminals of the active odour-specific subpopulation of KCs receive taste information and dopaminergic post-ingestive reinforcement signals (Aso et al. 2014, Aso & Rubin 2016, Bielopolski et al. 2019, Hige et al. 2015) which trigger intracellular changes that ultimately strengthen or weaken their ability to communicate with MBONs (mushroom body output neurons; Fig. 2). In short, coincident activity in the MBs drives synaptic plasticity and stores memories as modified KC-MBON connectivity. When the odour is reencountered, approach-promoting MBONs will fire more strongly and

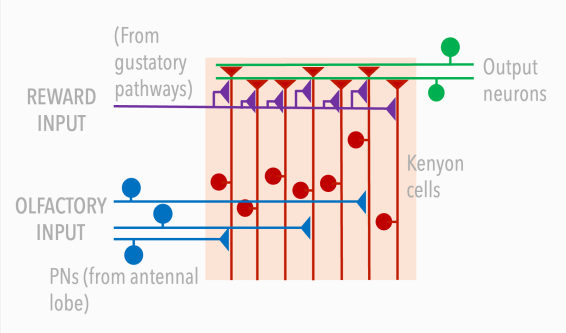


Figure 2 | Schematic mushroom body diagram. Odour reception activates a subset of KCs (red), and coincident reward input (purple) at these cells modifies activity at the corresponding KC-MBON synapses.

avoidance behaviours will be inhibited in response to the same pattern of odour-driven KC activity.

Bees can make use of many types of sensory information to discriminate between flowers (Rands et al. 2023). The MBs function as a general associative learning substrate, and information from other sensory modalities can be relayed from other brain structures to drive KC activity in their respective MB regions to produce associative memories (Buehlmann et al. 2020, Jesusanmi et al. 2024, Plath et al. 2017, Vogt et al. 2014). This can also allow the formation of multimodal associations (Yagi et al. 2016) which can improve the robustness of recognition when stimuli in each modality are low in intensity (Jordan et al. 2024, Gil-Guevara et al. 2022). That is not to imply that the mechanisms of associative learning in different sensory domains overlap fully (Gerber & Smith 1998) and much remains unknown about the mechanisms of multimodal learning in bees, particularly with spatial information. Generally speaking, though, bees are best at olfactory associative learning tasks but don't necessarily depend on these cues in order to learn to find food, and the mechanisms for doing so share some overlap (Vogt et al. 2014).

The role of cholinergic signalling in reward learning in the bee brain

To a significant extent, the insect associative learning pathway relies on cholinergic signalling.

Acetylcholine is a neurotransmitter that mediates sensory processing, cognition, and motor function in animals (Klinkenberg et al. 2011, Newman et al. 2012, King & Hunter 2002), and serves as the most abundant excitatory neurotransmitter in insects (Gauthier 2010). It functions by binding to acetylcholine receptors (AChRs), which are subdivided by and named after their relative sensitivity for nicotine and muscarine – nicotinic and muscarinic acetylcholine receptors (nAChRs and mAChRs), respectively. Both are expressed throughout the insect CNS (Dupuis et al. 2012, Silva et al. 2015, Trimmer 1995).

Each receptor type is also distinguished by its mechanism of action. nAChRs are ligand-gated ion channels that mediate fast, excitatory signalling (McGehee et al. 1995) and it is this form of cholinergic neurotransmission that largely brings about cognition, reward, and motor activity. nAChRs are heteromeric: they comprise five subunits, for which bees have 11 different genes (Jones et al. 2006, Sadd et al. 2015). Various other structural features can confer them with different pharmacological properties (Fasoli & Gotti 2015), making them a large and heterogenous family of receptors with varying sensitivities to different ligands (Lu et al. 2022, Tomizawa & Casida 2005). Given that these different receptor subtypes may be distributed unevenly around the body (Gauthier 2010) it is likely that different nAChR agonists may have slightly different pharmacological effects despite ostensibly sharing the same mechanism of action.

By contrast, mAChRs are metabotropic, G protein-coupled receptors, making them slower acting. mAChRs come in excitatory and inhibitory subtypes. Bees are thought only to express three types (Pilon et al. 2022), and they are present in much lower densities in the insect CNS than nAChRs (Trimmer 1995). A lot remains unknown about their function, especially in insects, but they seem to mediate synaptic plasticity and associated neurostructural changes, and play important neuromodulatory roles (Bielopolski et al. 2019, Manoim et al. 2022, Trimmer 1995).

The olfactory projection neurons mainly drive KC activation by releasing acetylcholine onto dendritic nAChRs (Fahrbach 2006, Yasuyama & Salvaterra 1999) and the KCs themselves communicate with the MBONs in the same way (Barnstedt et al. 2016, Gu & O'Dowd 2006). Kenyon cells also express mAChRs (Shih et al. 2018, Bielopolski et al. 2019). Hypothetically, any drug that can interfere with these signalling mechanisms could potentially influence the acquisition and/or recall of conditioned associations.

In mammals, highly nicotine-sensitive nAChRs are also expressed in dopaminergic neurons (DANs) that are important for encoding rewarding memories (Miwa et al. 2011, Biasi & Dani 2011, Biasi & Salas 2008). In the insect brain, the ongoing reinforcement signals that are continually provided to the MBs to tune plasticity within the KC–MBON network to direct appropriate behaviour are also provided by dopaminergic neurons (Waddell 2013). Additionally, nicotine also

seems to mediate dopamine release in the insect brain (Pyakurel et al. 2018, Shin & Venton 2018). It is therefore possible that cholinergic compounds might modify activity in insect DANs in the same way, directly affecting the reward input. Compounds that can usurp the reward-encoding activity of dopaminergic neurons can drive unconstrained drug approach behaviours, giving them the potential to become addictive (Jovanoski et al. 2023).

The biology of addiction

Dopamine is the critical neurotransmitter for encoding valence in mammals and insects. Animals have both appetitive and aversive populations of dopaminergic neurons (DANs). In *Drosophila*, and likely in bees too (Tedjakumala et al. 2017), those that signal to the MB are partitioned into anatomically separate populations (Waddell 2016). The DANs of mammals (Menegas et al. 2015, Avvisati et al. 2024) are organised in analogous ways. The rewarding dopaminergic neurons are far more numerous: *Drosophila* have about 10x more appetitive than aversive DANs (Li et al. 2020).

Appetitive DANs fire in response to novel, rewarding stimuli (Mirenowicz & Schultz 1994) – in insects, this begins to tune the MBON network – or in response to the learned cue (Schultz 2007, Saunders et al. 2018). The intensity of firing diminishes with increasing familiarity as the association and behavioural response becomes habitual (Kutlu et al. 2023). If reward is not delivered as expected after the cue, firing is inhibited. On the other hand, the activity of aversive DANs is generally suppressed by unpleasant experiences leading to a decrease in DA release. There are exceptions to this - some DANs respond with excitation to aversive events (Ungless et al. 2004, Brischoux et al. 2009, Matsumoto & Hikosaka 2009). However, generally speaking, both populations of DANs respond to reward prediction error such that rewards and punishments are signalled along a single axis on which dopamine release acts as a rewarding teaching stimulus (Schultz 2007).

The neural substrate of nicotine addiction has not yet been mapped in detail and we lack knowledge of the expression patterns of nAChRs across the appetitive and aversive DANs in the bee brain. However, nicotine observably promotes dopamine release in the brain (Pyakurel et al. 2018). It evades degradation by acetylcholinesterase (AChE) causing it to desensitise nAChRs (Quick & Lester 2002) and thus the reward signals are amplified and prolonged. Drugs of abuse therefore synthetically engage and usurp the reward system to encode stubborn, salient, super-rewarding memories (Hyman 2005, Hyman et al. 2006, Jovanoski et al. 2023) and, by extension, drug-seeking habits.

Lots of research on nicotine addiction reveals that repeated drug exposure causes the brain and body to adapt to its presence through compensatory changes in the brain's signalling mechanisms. Recurrent nicotine exposure causes increased expression of nAChRs and differences in their subunit composition (Biasi & Dani 2011) which may affect their sensitivity to ACh and nicotine. These are homeostatic adaptations that have a stabilising effect on cholinergic neurotransmission but with knock-on effects on the function of dopaminergic, glutamatergic, and opioid pathways that are involved in reward and motivational state (Perez et al. 2012, Zhang & Sulzer 2004, Changeux 2010). One consequence of this is that sensitivity to natural rewards can be blunted while responsiveness to nicotine is potentially increased (Changeux 2010). Another is that animals become dependent on the presence of the drug to maintain their homeostatic balance, and motivation to acquire the drug shifts from positive to negative reinforcement (Koob et al. 2014, George 2019). Once drug dependence has been established, if the drug is withheld, an animal enters a malaise state (Malin et al. 1992). Under withdrawal drug seeking increases progressively in a bid to re-establish homeostasis (Koob 2008), and somatic symptoms are manifest. In rats, these include trembling, teeth chattering, yawning, and abnormal grooming (Gipson et al. 2020, Malin et al. 1992).

Addiction itself is a condition that tends to be behaviourally characterised as a shift from volitional to compulsive drug use, and is defined by the DSM (American Psychiatric Association 2022) by its associated behavioural symptoms which might broadly be summarised as unconstrained drug approach behaviours. In animal models, these may be observed as increased rate of intake over time, self-administration despite punishment, persistent attempts to acquire the drug from a learned source, and increased drug seeking under withdrawal (Deroche-Gamonet et al. 2004, Biasi & Dani 2011, Biasi & Salas 2008, Jovanoski et al. 2023).

Assaying addiction in insects

Rodent models have been fruitful in addiction research. Studies have identified addictive molecular and cellular changes in the rodent brain (Miwa et al. 2011, Changeux 2010, Biasi & Dani 2011) as well as more comprehensive behavioural symptoms including the development of tolerance (Falkeborn et al. 1981, Rosecrans et al. 1989,

Collins et al. 1988), withdrawal behaviours (Malin et al. 1992, O'Dell et al. 2004), and reduced sensitivity to natural rewards and punishments (Epping-Jordan et al. 1998, Pergadia et al. 2014). Whereas humans self-administer various addictive drugs, including orally; the majority of these experiments involved entraining animals to do so intravenously or depriving them of food and/or water in order to prevent initial rejection due to pre- and post-ingestive aversion. The voluntary development of compulsive drug-seeking behaviour has therefore been difficult to model in animals (Hyman et al. 2006).

In theory, invertebrates ought to be tractable systems too. Their brains are much smaller but share largely conserved mechanisms of reward with mammals (Waddell 2013) and the neural architecture of associative learning and memory is also analogous in its structure and function (Scaplen & Kaun 2016, Aso et al. 2014, Hourcade et al. 2010). Despite this, they have been relatively understudied (Søvik & Barron 2013). A body of work examines the effects of various nectar compounds on bee foraging behaviours, but relatively few studies are carried out with an addictive focus. The invertebrate addiction literature remains quite fragmented, employing sparse and preliminary combinations of drugs, organisms, doses, and experimental paradigms.

Some groundwork has aimed to confirm that various drugs of abuse also act as reinforcer in invertebrates using associative learning tasks of various sorts. Morphine ingestion seems to have been conditioned in ants (Entler et al. 2016), and direct delivery of addictive drugs to the hemolymph in an otherwise avoided environment appears to induce preferential visitation in place preference assays using crayfish (Nathaniel et al. 2009, Kusayama & Watanabe 2000, Panksepp & Huber 2004). Planarians climb nicotine gradients on a petri dish in a way that indicates unconditioned reward (Sellings et al. 2013). Bees were also found to waggle dance more readily and enthusiastically in response to food when simultaneously treated with cocaine, apparently independently of any locomotor influence (Barron et al. 2009). However, all of these experiments also either involved training the animals to self-administer the drug or some form of enforced topical application. Would they do this if not initially coerced to interact with the drug? An alternative tack taken to understand drug reward in invertebrates has been to use mass spectrometry to assay the concentration of different biogenic amines in the brain as close as possible to the time at which the animal hypothetically experienced the reward (Søvik et al. 2013, Entler et al. 2016). This approach has its problems too – firstly, because reward signalling depends on transient, localised changes in neurotransmitter release yet whole brain sampling quantifies both extracellular and intracellular pools of biogenic amines with temporal imprecision, and the idea that octopamine signals reward and dopamine punishment is overly simplistic anyway (Waddell 2013, Burke et al. 2012).

Compulsive drug-seeking comes about through the development of a super-rewarding memory via repeated hedonic interactions with the compound (Hyman et al. 2006). Ideally it should therefore be assayed in a paradigm that allows room for it to develop out of voluntary *ad lib* drug exposure rather than in artificial ways that require behavioural shaping and/or enforced drug delivery mechanisms.

Voluntary hedonic interactions of invertebrates with drugs of abuse have more rarely been studied. Ethanol in food elicits preferential feeding in relatively unconstrained choice tests despite seeming to slow learning in honeybees during olfactory conditioning (Mustard et al. 2008, 2019, Sokolowski et al. 2012). Drosophila also show preferences for feeding on ethanol-laced food (Ogueta et al. 2010, Koyyada et al. 2018). This appears to be true regardless of pre-exposure and, as authors suggest, the drug's nutritional value. In feeding preference tests, bees can also prefer nicotine (Singaravelan et al. 2005, Baracchi et al. 2015, 2017). In general, it has been hard to conclude that these changes have an addictive cause because they have not been tested for a compulsive quality.

Following repeated exposure to drugs of abuse, documented addictive behavioural changes in invertebrates include withdrawal (Barron et al. 2009, Ostap-Chec et al. 2021) and the development of tolerance (Rothenfluh & Heberlein 2002, Park et al. 2017, McClung & Hirsh 1998, Søvik et al. 2013). But compulsive drug seeking in an insect species has not yet been shown. Is this addictive symptom unique to mammals?

Bees naturally encounter these compounds in nectar (Mustard 2020) and have poor bitter taste detection thresholds (Kessler et al. 2015, Tiedeken et al. 2014, Wright et al. 2010). It is therefore possible that bees are one of the only animals that, through voluntary ingestion of ecologically relevant drug doses, might come under natural conditions to preferentially seek addictive drugs in the same way that humans do.

Ecological distribution of cholinergic nectar drugs

The tissue concentrations of defensive alkaloids have often been sampled from flowers and vegetative tissues, but comparatively little is generally

known about their nectar presence. If they influence cholinergic signalling and are expressed in nectar at opportune concentrations, many of these compounds could potentially be addictive to foraging bees.

I explored the literature to try and pull together some candidate drugs for behavioural studies. The search was focussed on natural nAChR or mAChR agonists or antagonists, or AChE inhibitors; with a known presence in floral tissue and/or nectar, and also looked for studies documenting their behavioural effects in mammals or invertebrates (Table 1). Some were excluded because reports indicated they were synthesised only in plants unlikely to be pollinated by bees (erythroidine, hygrine, piperine), and others due to a lack of available information (arecaidine, belladine, corynoline, cotinine, cuscohygrine, guavacoline, guvacine, linearilin, linearilobin, lupinine, lycactonine, myosmine, physostigmine, salsoline, sparteine, tubocurarine).

Table 1 | The ecological distributions and behavioural effects of floral cholinergic drugs. n.d. = not determined

Drug	MoA	Plant species	Presence in nectar/ floral tissue	Nectar conc.	Cognitive and/or behavioural effects	References
Anabasine	nAChR agonist	*Nicotiana* sp.	Present in nectar	∼1–40 µM	Feeding deterrent to honeybees and sunbirds at higher natural concentrations. In sunbirds, decreased foraging performance. Increases midbrain dopamine levels in humans.	Adler et al. (2006), Detzel & Wink (1993), Hoffman & Evans (2013), Kasiotis et al. (2020), Singaravelan et al. (2005), Tadmor-Melamed et al. (2004)
Anatabine	nAChR agonist	*Nicotiana* sp.	Present in *Nicotiana glauca* flowers; not yet detected in nectar	n.d.	No studies in bees. Increases dopamine release in the nucleus accumbens but conditioned place aversion in rats.	Kasiotis et al. (2020), Marusich et al. (2017)
Arecoline	nAChR and mAChR agonist	*Areca catechu* (betel nut)	Some in flowers; more in male than female. Nectar presence not investigated	n.d.	Bees: low doses slightly improved learning and memory, addictive gene exp patterns; high doses caused impaired cognition and locomotor hyperactivity. Rats: dose-dependent changes to memory; altered taste bud cell morphology and reduced sugar sensitivity.	Liu et al. (2025), Li et al. (2025), Molinengo et al. (1995), Ono et al. (1995), Peng et al. (2016), Wu et al. (2019)
Coniine	nAChR agonist	*Conium maculatum, Sarracenia flava, Aethusa cynapium*	Present in flowers and secretory ducts. Sharp increase at flowering, pollination, and fertilisation	n.d. Levels high in flowers compared to other tissues	Not investigated in bees. Diptera pollinate poison hemlock (*Conium maculatum*) in large numbers, and are thought to find it attractive (Nitao, 1987).	Hotti & Rischer (2017), López et al. (1999), Nitao (1987), Vetter (2004)

Table 1 (continued)

Drug	MoA	Plant species	Presence in nectar/ floral tissue	Nectar conc.	Cognitive and/or behavioural effects	References
Cytisine	nAChR agonist	*Cytisus laborinum*; some Fabaceae genera (*Laburnum, Anagyris, Thermopsis, Cytisus, Genista, Retama, Sophora*), and Gymnocladus trees	Not investigated/ reported in nectar. Lots in flowers of various sp including *Retama sp., Gonocytisus pterocladus, Templetonia incana, Genista sp., Petteria ramentacea, Spartium junceum*	n.d.	Hypothesised to help recruit honeybees to the nectar of *Sophora microphylla*, which is "very attractive to honeybees" (Clinch et al., 1971), though presence in nectar hasn't been explicitly identified. Learning and memory enhancements in rodents and zebrafish at low doses.	Belsito et al. (2008), Braida et al. (2014), Clinch & Forster (1972), El-Shazly et al. (1996), Greinwald et al. (1992, 1996), Makara et al. (2018), Tosun & Çiğdem Akyüz (1999), Veen et al. (1992), Wüstenberg & Grünewald (2004)
Galantamine	nAChR agonist and AChE inhibitor	Family *Amaryllidaceae*, inc. *Amaryllis, Lycoris, Hippeastrum, Ungernia, Leucojum, Zephyranthes, Narcissus, Galanthus, Hymenocallis*, and *Haemanthus*	No reported nectar presence. Present in flowers of various sp. Levels may be lower in blooming flowers than budding/ wilting (Jiang et al., 2011)	n.d.	No reported effects on bees. Some evidence that can enhance learning and memory in mammals, especially when impaired	Jiang et al. (2011), Matthews et al. (2013), Park et al. (2021), Souza et al. (2011), Sinan et al. (2020), Tsvetkova et al. (2014), Weible et al. (2004)
Hyoscamine	mAChR antagonist	Certain *Solanaceae* plants, notably jimsonweed, henbane, mandrake, angel's trumpets, tomato, the sorcerers' tree, and deadly nightshade	High quantites in *Hyoscamus albus* flowers. Also found in *Datura* flowers	n.d.	Can cause memory impairment. Can condition appetitive or aversive odour responses by locusts depending on their phase	Al-Snafi (2017), Bazaoui et al. (2012), Laksty-gal et al. (2019), Simões et al. (2013)

Table 1 (continued)

Drug	MoA	Plant species	Presence in nectar/ floral tissue	Nectar conc.	Cognitive and/or behavioural effects	References
Lobeline	Mixed agonist–antagonist at nAChRs	Various, esp in genus *Lobelia*, inc. *L. inflata, L. tupa, L. siphilitica, Lobelia chinensis*; and *Hippobroma longiflora*	Reported to be present in the 'aerial parts' of Lobelia inflata	n.d.	Mixed but often mildly enhancing cognitive effects in humans and rats. Feeding preference in a caterpillar but seems to taste bitter to drosophila. Rats don't seem to self-administer	Damaj et al. (1997), Decker et al. (1993), Dussourd (2003), Dwoskin & Crooks (2002), Levin (1996), Terry et al. (1996), Turchi et al. (1995), Sellier et al. (2011), Marella et al. (2006), Weiss et al. (2011)
Methyllycaconitine (MLA)	nAChR antagonist	Found in at least 30 *Delphinium* species as well as in *Consolida ambigua* and *Inaularoyaleana*. Principal toxin in *Delphinium brownii*	Present in *Delphinium* nectars. *Delphinium barbeyi* nectar levels are low relative to those of other tissues	$\sim$ 28-56nM in *Delphinium* nectar	Mixed (probably dose-dependent) effects reported on cognition in rats. Little effect on honeybee learning or STM found when injected into brain. Possible impairment to honeybee LTM. Metabolised quickly by honeybees.	Boccia et al. (2010), Davis & Gould (2006), Dobryakova et al. (2015), Gauthier (2010), Gauthier et al. (2006), van Goethem et al. (2019), Manson et al. (2013), Milienne-Petiot et al. (2018), Raybuck & Gould (2010), Vago & Kesner (2007), Vidkjær et al. (2021)
Nicotine	nAChR agonist	*Nicotiana* spp., *Duboisia hopwoodii*	Known to be present in *Nicotiana* nectar	Typically 1-30μM (but can be higher)	May enhance learning and floral fidelity in bumblebees. Feeding deterrent to bees at high concentrations, but attractant at low. May reduce larval, but not adult, survival	Baracchi et al. (2017), Detzel & Wink (1993), Human et al. (2014), Kessler & Baldwin (2007), Köhler et al. (2012), du Rand et al. (2017), Singaravelan et al. (2005, 2006), Tadmor-Melamed et al. (2004), Thany & Gauthier (2005)
Nornicotine	nAChR agonist	Some *Nicotiana* plants. One of the four main alkaloids in *Nicotiana tabacum*	Small amounts in flowers – possibly specifically and exclusively. Enzymatic evidence suggests could be present in nectar	n.d.	Reinforcing effects in mammals similar to nicotine and leading to preferences and self-administration. No studies in insects	Bardo et al. (1999), Caine et al. (2014), Desai et al. (2016), Harris et al. (2015), Kasiotis et al. (2020), Liedschulte et al. (2016), Majdi et al. (2019), Marlin et al. (2014), Tomizawa & Yamamoto (1992)

Table 1 (continued)

Drug	MoA	Plant species	Presence in nectar/ floral tissue	Nectar conc.	Cognitive and/or behavioural effects	References
Pilocarpine	mAChR agonist	Genus *Pilocarpus*	Present throughout *Pilocarpus microphyllus* tissue, including flower	n.d.	Increases volume of bee neuropil in similar way as foraging experience. Improves nestmate recognition by young honeybees. Low doses may improve learning in older rats	Dobrin et al. (2011), Ismail et al. (2006, 2008), Prediger et al. (2006)
Scopolamine	mAChR antagonist	*Solanaceae* sp. inc. *Hyoscyamus niger, Datura, Brugmansia, Belladonna, Mandragora officinarum*, and *Duboisia*	Yes, and in the *Hyoscyamus albus* and *Datura* floral tissue	9μM – 1.3mM	Generally had detrimental/no effect learning and memory in bees and mice. In bees, enhanced the rate of synaptic pruning in foragers; may be phagostimulatory; increased attacks on nestmates	Boros et al. (2010), Bazaoui et al. (2012), Feiro & Gould (2005), Gauthier et al. (1994), Hefco et al. (2003), Ismail et al. (2008), Kerchner et al. (2015), Lakstygal et al. (2019), Liao et al. (2019), Lozano et al. (2001), Sculfort et al. (2021), Sharma et al. (2021), Ullrich et al. (2016)
Sparteine	mAChR agonist	*Lupinus* sp., *Cytisus* sp. (scotch broom), *Spatium junceum* (broom), *Retama* sp.	Present in *Cytisus* and *Lupinus aschenbornii* flowers	n.d.	Little influence on learning or STM. Feeding deterrent in moth larvae and some grasshoppers – phagostimulant in others	(El-Shazly et al., 1996; Hernández et al., 2011; Picaud et al., 2003; Shields et al., 2008)
Trigonelline	AChE inhibitor	Widespread plant hormone. Higher levels in certain sp., including coffee (esp arabica), *Murraya paniculata*, *Mirabilis jalapa*	Present in linden (*Tilia cordata*) and faba bean (*Vicia faba*) nectar. In floral tissue of various sp, inc. *Leucaena leucocephala*; and Arabica and Conilon coffee flowers.	∼15-180μM	Generally thought to impair bee learning and/or memory, though evidence anecdotal	(Ashihara & Watanabe, 2014; de Abreu Pinheiro et al., 2021; Lande et al., 2019; Mathur & Kamal, 2012; Ogita et al., 2014)

Our knowledge of the nectar presence of these compounds remains relatively sparse. More work needs to be done to identify nectar drug concentrations and then the cognitive effects of field-relevant doses can be investigated. Ideally, it would also be helpful to quantify the accompanying distribution of nectar nutrients which could play an important role in how pollinators experience these compounds in nectar through taste mechanisms (von Frisch 1967, Gegear et al. 2007, Köhler et al. 2012, Cocco & Glendinning 2012) and could also influence the development of learned preferences (Chittka & Schürkens 2001). These data may then also help to identify if neuroactive secondary metabolites have independently evolved to manipulate pollinator behaviour within various plant lineages.

Reported behavioural effects are generally mixed, and relatively little of the behavioural work was done in bees or other invertebrates. It seems likely that these effects will be highly dose-dependent (Calabrese 2008, Grasing et al. 2022) whereas the mechanism of action of a drug, at least when considered in isolation, might not have much predictive power over its behavioural output.

Despite those sizeable caveats, there may be some slight but discernable trends. For instance, nAChR agonists seemed to produce the most consistent enhancements to learning and/or memory. In comparison, the behavioural effects of mAChR agonists have rarely been investigated and are less clear, but at low doses they may have learning-enhancing effects. Antagonists of either receptor type and acetylcholinesterase inhibitors tended to have little to no effect in the tested experimental contexts, with the possible exception of scopolamine.

What we currently know about addiction to cholinergic drugs in bees

Addiction in bees to nectar laced with these drugs is a largely uncharted research area, and most work that bears on the question was not conducted with an addictive focus.

Some studies investigate whether nicotine and neonicotinoids can influence normative reward valuation in bees using feeding preference assays, in which animals are presented with a choice between drugged and undrugged solutions of equal nutritional value. Nicotine is often, though not always (Tiedeken et al. 2014, Palmer-Young et al. 2017) preferred (Singaravelan et al. 2005, Köhler et al. 2012, Baracchi et al. 2015, 2017) at nectar-relevant concentrations. The reasons for these discrepancies are currently unclear but could be linked to differences in the ability of bees to move freely – bees in the former group (Tiedeken et al. 2014, Palmer-Young et al. 2017) were not able to fly – or the duration of the test phase, if, as we might expect, motivation to ingest nicotine is in flux and preferences are temporally unstable. Low doses of neonicotinoids also appear to produce preferences by bees in choice assays (Arce et al. 2018, Kessler et al. 2015, Tiedeken et al. 2014). Scopolamine reportedly elicits initial feeding bouts that are longer in duration (Sculfort et al. 2021) which may indicate it has a reinforcing quality, but the concentration used in this work ($\sim$30mM) was very high, and at field-relevant concentrations this perhaps may not hold (Detzel & Wink 1993).

Some have tried to assay the pharmacological influence of cholinergic drugs on learning and memory processes in bees more directly, but most often have delivered the drugs orally, topically, or via injection into the brain at time points independent of the food delivery. Perhaps unsurprisingly, sustained, reward-irrelevant nAChR activation tends not to improve the success of associative learning or memory (Hassani et al. 2008, Decourtye et al. 2003, Decourtye, Armengaud, Renou, Devillers, Cluzeau, Gauthier & Pham-Delègue 2004, Decourtye, Devillers, Cluzeau, Charreton & Pham-Delègue 2004, Piiroinen & Goulson 2016, Stanley et al. 2015, Aliouane et al. 2009, Ramirez-Romero et al. 2008, Williamson & Wright 2013, Liu et al. 2025) (though see Williamson et al. (2013), Thany & Gauthier (2005)). In the field drugs will be experienced in food as part of the learning experience, hypothetically acting with temporal specificity to reinforce the associations. When offered in reward, nicotine does appear to enhance learning in free-flying bees and encode more persistent memories (Baracchi et al. 2017) though the neonicotinoids imidacloprid and thiamethoxam did not in an olfactory conditioning assay (Wright et al. 2015). Questions and ambiguities remain but it nonetheless seems that some of these compounds may have the potential to enhance the processes of food learning and/or memory in a way that would be expected if the food were more highly rewarding.

Liu et al. (2025) observed that forced pre-exposure to arecoline for 6 days induces addictive gene expression patterns in the bee brain. Whether the reinforcing qualities of cholinergic nectar drugs translate to addictive behavioural patterns of self-administration and the development of drug dependence has not been tested. In the study by Baracchi and colleagues (2017), a subset of bees persisted in visiting artificial flowers even after the drug-laced food had been withdrawn. This could have various causes, not all addictive, but it's possible that could have been an expression of compulsive drug-seeking behaviour

following the development of a super-rewarding memory (Hyman et al. 2006). No studies have yet directly addressed whether oral exposure to drugged nectar can give rise to compulsive drug seeking and/or withdrawal in bees.

This thesis

The work presented here targets this question using compounds in nectar that affect cholinergic signalling. Most of these experiments use nicotine as a model drug, which is a nAChR agonist naturally encountered by bees (Fig. 3) in certain *Solanaceae* nectars, most notably *Nicotiana* (Adler et al. 2006, Kessler et al. 2012, Singaravelan et al. 2005, Tadmor-Melamed et al. 2004, Tiedeken et al. 2014).

Chapter 2 uses two existing assays to explore whether food laced with a nectar-relevant concentration of nicotine is preferred by bees, how it affects their learning and memory, and whether these effects change with long-term drug exposure in food followed by a period of abstinence.

The choice experiment protocol used in chapter 2 (experiment 2a) assays the feeding decisions in aggregate of groups of bees across a set timeframe. It is therefore well suited to use with test solutions of a fixed and relatively salient difference in quality. Similarly, olfactory conditioning of the PER (experiments 2bi and 2bii) relies on averaging responses across subjects and works best in the absence of heterogeneous effects. To assay perceived distinctions in reward value that may be slight, in flux with an animal's homeostatic state, and dependent on individual variation, I developed a new method for continuous monitoring of individual feeding decisions. In chapter 3 I detail this method and present pilot test data (experiment 3) which validate the deterrent quality of a moderate concentration of quinine in sucrose using a variety of metrics.

A hallmark behaviour associated with drug dependence is withdrawal-induced drug-seeking. To corroborate my previous findings, I used the new setup to measure whether nicotine feeding was altered at the beginning of the experiment if bees were pre-exposed to and withdrawn from the drug. To identify how preferences develop and test whether bees could become addicted to nicotine through voluntary oral exposure, I also monitored the feeding decisions made by drug-naive bees over 24 h. These data are presented in chapter 4 (experiment 4a). I had also predicted that any compound that influences cholinergic neurotransmission could potentially be addictive to foraging bees. In a second experiment presented in chapter 4 (experiment 4b), I screened a selection of other nectar-relevant drugs at their known or assumed nectar concentrations for their potential to induce similar behavioural changes in bees through pre-exposure and withdrawal. I chose several compounds that target AChRs directly, plus caffeine, which is known to act indirectly to influence cholinergic signalling and pharmacologically amplify the rewarding properties of nectar sugars.



Figure 3 | **Bees foraging from *Nicotiana* flowers**. Left: bumblebee (from reddit); right: honeybee (flickr).

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Chapter 2 | The influence of nicotine-laced nectar on feeding preferences and food reward learning in honeybees

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Abstract

Nicotine and other cholinergic drugs are naturally present in certain floral nectars. These compounds have the capacity to interact with nAChRs in the bee nervous system and are thought to influence reward signalling, potentially in a way that could make bees addicted. However, studies investigating their effects on reward learning and food choice behaviour in bees have produced inconclusive results. Here, we use a combination of behavioural assays to investigate how exposure to nicotine in food may influence these processes. Using a 24 h two-way feeding choice experiment, we first test whether groups of honeybees show innate preferences for nicotine-laced nectar over equimolar sucrose, and whether this changes with drug pre-exposure and withdrawal. Bees only developed robust feeding preferences in these tests when they had been pre-exposed to the drug for 72 h and subsequently undergone a period of abstinence. Using analogous treatment schedules, we then performed a closer examination of the mechanisms of state-dependent drug reinforcement in an olfactory conditioning task using nicotine-laced reward. Nicotine had equivocal effects on naive bees, but those that had been pre-exposed to it showed potentially addictive behavioural patterns of responding to either reward type.

INTRODUCTION

Many flowering plants are animal-pollinated. In combination with visual and olfactory stimuli, which provide pollinators with cues for locating and learning flowers in a competitive landscape, floral nectar facilitates associative learning. Preferred rewards will be more reinforcing and the resultant associations easier to learn. We therefore expect nectar to be adapted to these feeding preferences to facilitate pollinator recruitment and retention.

Secondary metabolites (SMs) are bioactive compounds that may be synthesised in various plant tissues to serve specialised ecological functions. They are often used to poison or repel herbivores, pathogens, and microbes (Teoh 2016) but are sometimes also present in floral nectar (Barberis et al. 2023, Stevenson et al. 2016). Toxicity of nectar could undermine its primary role in pollinator attraction, so this has been regarded widely as an evolutionary paradox. It could represent an awkward compromise between attracting pollinators and defending against herbivores and nectar robbers. However, nectar SMs have been hypothesised to serve adaptive functions within plant-pollinator interactions in several ways: they may be used to recruit specialist pollinators with evolved tolerance or preference for these nectars, medicate pollinators against pathogens and parasites, and preserve nectar quality (reviewed by Stevenson et al. (2016)). Additionally, some of these compounds can interact directly with animal nervous systems (Mustard et al. 2020) by mimicking, blocking, or modulating neurotransmitters; or otherwise altering the flow of ions at ion channels. This gives them the potential to influence behaviour.

Nicotine is a pharmacologically active SM that bees primarily encounter in *Solanaceae* nectar at sublethal, non-deterrent concentrations in the range of 0.5 - 30μM (Singaravelan et al. 2005, Tadmor-Melamed et al. 2004, Kessler et al. 2012, Tiedeken et al. 2014). Bees show some adaptations to dietary nicotine and are one of the few insects able to detoxify it (du Rand et al. 2015), but, when ingested, it is transported to the brain in the hemolymph and accumulates in tissues throughout the body (du Rand et al. 2017). The drug mimics acetylcholine (ACh), which is important for sensory processing, cognition, and motor function in animals; and is the principal excitatory neurotransmitter in the insect brain (Gauthier 2010). ACh functions through combinations of nicotinic and muscarinic acetylcholine receptors (nAChRs and mAChRs), both of which are expressed broadly in insect neural and non-neural tissue. Nicotine agonises nAChR subtypes with different affinity. In the mammalian brain, it binds particularly to those found in dopaminergic neurons which are required for the formation of reward memories (Miwa et al. 2011, Changeux 2010, Subramaniyan & Dani 2015). In insects, cholinergic signalling via nicotine-sensitive nAChRs also affects the formation of reward memories in various ways. For instance, Kenyon cells (KCs) receive input from olfactory projection neurons which are mostly cholinergic (Fahrbach 2006), and KCs themselves rely largely on ACh for neurotransmission to the mushroom body output neurons (MBONs) (Barnstedt et al. 2016). Cholinergic MBONs elicit approach behaviours (Aso et al.

2014, Cognigni et al. 2018). By pharmacologically influencing circuits such as these, nicotine-laced nectar might give plants the ability to usurp the bee's reward encoding system and increase the rate at which pollinators specialise on conspecific flowers. Unlike ACh, nicotine is not hydrolysed by acetylcholinesterase, causing it to desensitise receptors and persist in synapses. With repeated cycles of exposure the brain adapts to the continual presence of nicotine by upregulating nAChRs (Buisson & Bertrand 2002, Govind et al. 2009, Christen et al. 2016) and modifying their subunit composition (Miwa et al. 2011, Walsh et al. 2008). Behaviourally, such neuroadaptations can lead to addictive cycles of drug seeking to maintain homeostasis.

Some work investigates the influence of nicotine on the nectar preferences of naive bees. Caged and free-flying honeybees and bumblebees seem to find nicotine-laced food aversive at high concentrations and potentially attractive at lower, more field-relevant levels (Singaravelan et al. 2005, Köhler et al. 2012a, Tiedeken et al. 2014, Baracchi et al. 2015, 2017). Evidence of preferences for low concentrations is generally weak, however, and sometimes inconsistent (Palmer-Young et al. 2017a, Köhler et al. 2012a). Low levels of neonicotinoids ('new nicotine' – synthetic nAChR agonists) in food may also generate feeding preferences for drugged food over timescales ranging from 24 h to 10 days (Kessler et al. 2015, Arce et al. 2018). These assays imply that bees could prefer feeding on cholinergic nectar in an ecological setting, but did not provide field-relevant volumes of reward, and do not manage to examine directly the influence of nicotine-laced food on reward learning and memory or its potential to generate addiction.

Olfactory conditioning of the proboscis extension reflex (PER) has been used to assay the pharmacological influence of cholinergic agonists on honeybee learning and memory. In these studies, drugs were introduced orally, topically, or via injection into the brain at time points independent of the food delivery – typically prior to conditioning – in order to isolate an effect of sustained nAChR activation on learning and recall. However, in the field, drugs will be experienced in reward, hypothetically serving as reinforcers. Much of this work reports impairment to learning and memory processes (Hassani et al. 2008, Decourtye et al. 2003, 2004a,b, Piiroinen & Goulson 2016, Stanley et al. 2015, Aliouane et al. 2009) though memory effects have tended to be a little more ambiguous (Thany & Gauthier 2005, Mustard et al. 2023). Bees in acute treatments were also often dosed with drug levels exceeding those found in the field. Additionally, depending on the experimental timescales, those that were treated chronically with these potentially addictive substances prior to testing could have experienced withdrawal effects on their neural function, physiological state and/or baseline motivation levels that could confound interpretations about cognitive changes. In summary, it is difficult to conclude from this work how nAChR agonists might influence reward learning or memory when encountered in nectar.

To date, no studies have explicitly screened nicotine-laced nectar for an ability to produce addictive behaviours in bees. Baracchi et al. (2017) found that nicotine in reward offered by artificial flowers tended to enhance forward learning in bumblebees and subsequently impair reversal learning such that they were more faithful to the flowers that had previously provided higher drug doses. The timescales of this experiment were not reported, and these results could be interpreted in various ways. However, it seems to indicate that nicotine experienced while foraging can bias and interfere with normative reward valuation.

We used two behavioural assays to tackle this set of questions. First, using a choice test over 24 h (experiment 2a), we screened food laced with field-relevant levels of nicotine for reinforcing properties in groups of bees in different states of chemical homeostasis by indexing their preference for it. Bees were either nicotine-naive, pre-exposed to it for 72 h, or pre-exposed for 72 h and withdrawn for a further 24 h; and were then either given sucrose only for 24 h ('no choice') or a two-way feeding choice with a nicotine-laced option. If nicotine were inherently reinforcing, we expected to see bees in choice treatments (*Test*) expressing positive preference indices, including in the naive group. We also compared preference between bees in the choice groups of different pre-exposure treatments. We predicted pre-treatment type would change the way nicotine was valued (*Pre-treatment* $\times$ *Test*) such that preferences were strongest in the withdrawal group.

Using analogous exposure paradigms, in experiments 2bi and 2bii we then examined more closely the mechanisms by which nicotine can generate preferences, and whether these depend on drug pre-exposure and/or homeostatic state. To do this, we tested how field-relevant doses of nicotine experienced in reward during 6-trial massed olfactory conditioning could modify the processes of learning and memory using naive vs drug pre-exposed bees. We predicted that if nicotine-laced reward were perceived as more valuable, it might accelerate learning during conditioning (*reward* $\times$ *trial*) or improve its ultimate success (*reward*), and/or improve the overall success of recall in the memory tests (*reward*, or *re-*

ward × *test*). If these effects were pre-exposure-dependent, we expected to see them interacting with pre-exposure type, as they might if only bees that are either naive to the drug or sensitised to it experience accelerated *pre-exp* × *reward* × *trial* and/or more successful (*pre-exp* × *reward*) learning, and/or if bees in the NS group were to experience impairments to either process. Pre-exposure-dependent reinforcement would also be detected similarly in the memory data as *pre-exp* × *reward* or *pre-exp* × *reward* × *test* interactions. Bees in the nicotine pre-exposed groups were placed in different putative motivational states during learning and memory testing between experiments: in experiment 2bi, they were exposed to the drug chronically prior to learning and withdrawn during the 24 h memory tests, whereas in 2bii, the 'intermittent' experiment, bees were deprived of nicotine before the learning task. We predicted that, if nicotine's influence on learning and/or memory were state-dependent, any of the above effects would be stronger when bees were withdrawn during the task (experiment 2bii), and a potential influence would also be more detectable in the memory data when bees were withdrawn at this time point (2bi).

METHODS

Experiment 2a (Preference assay)

Capture and restraint of subjects

Foraging honeybees (*Apis mellifera buckfast* or *carnica*) were captured on return to the hives in glass vials of five bees. They were then chilled briefly on ice until immobile and transferred into ventilated plastic boxes (145 x 130 x 60 mm) in groups of 20 individuals (Fig. 1, each representing one sampling unit). Approximately 30 boxes of bees were used per treatment group. The boxes had holes of 10 mm in diameter either side for the insertion of feeding tubes, which were 2 ml centrifuge tubes with a series of four holes, 2 mm in diameter, drilled along the side. Bees were initially left in the boxes on the bench for 1 h with no food to acclimatise to the caged environment.

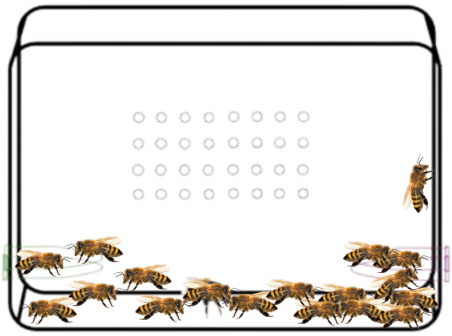


Figure 1 | **A feeding cage**. Bees were held in groups of 20 within ventilated plastic boxes, and given access to the test solution in feeders made from modified eppendorf tubes. Each box of bees constituted one sampling unit.

Solutions

All solutions were made using deionised water and reagent grade sucrose. Stock nicotine solutions (25 mM) were produced by dissolving nicotine ditartrate dihydrate salt (Sigma Aldrich; hereafter, 'nicotine') in 1 M sucrose.

Group assignment and pre-treatment of subjects

Three different pre-exposure treatments were used: no pre-exposure, 72 h drug pre-exposure, and 72 h drug pre-exposure followed by a 24 h period of withdrawal (WD). Bees within each of these cohorts were either given a choice between sucrose and nicotine during the test phase or assigned to a 'no choice' control treatment (1 M sucrose vs 1 M sucrose). Cages of bees were randomly assigned to these groupings, which are summarised in Table 1.

Drug pre-exposed bees were provided 72 h of *ad libitum* access to 1 M sucrose laced with 25 μM nicotine, which was delivered in the feeding tubes. Those in the withdrawal treatments were subsequently provided with 1 M sucrose for an additional 24 h prior to testing. At the same time each day, fresh tubes were inserted and the number of surviving bees in each cage logged. Daily food consumption per cage was recorded by weighing the tubes before and after feeding and subtracting the amount evaporated from control tubes.

Table 1 | Experiment 2a (preference assay) treatment designations

Treatment	Label	Pre-exposure phase	Test phase
Naive, no choice	Naive0	NA	1M suc vs 1M suc
Naive, choice	NaiveX	NA	1M suc vs 1M suc + 25μM nicotine
Pre-exp, no choice	Pre-exp0	1M suc + 25μM nicotine for 72h	1M suc vs 1M suc
Pre-exp, choice	Pre-expX	1M suc + 25μM nicotine for 72h	1M suc vs 1M suc + 25μM nicotine
Pre-exp + WD, no choice	WD0	1M suc + 25μM for 72h, then 24h 1M suc	1M suc vs 1M suc
Pre-exp + WD, choice	WDX	1M suc + 25μM for 72h, then 24h 1M suc	1M suc vs 1M suc + 25μM nicotine

Preference test

In the test phase, bees of each pre-exposure type were presented with a choice between either 1 M sucrose (S) vs 1 M sucrose + 25 µM nicotine (N), or 1 M sucrose vs 1 M sucrose according to their group (Table 1) for 24 h. These positions were counterbalanced across cages within each treatment. The feeders were weighed before and after the test phase, and amount evaporated was subtracted from these mass change values using data from evaporation control cages containing no bees. Preference indices for the nicotine-laced solution were calculated using the following formula:

$$\mathrm{PI} = \frac{\text{Mass consumed}_\mathrm{N} - \text{Mass consumed}_\mathrm{S}}{\text{Mass consumed}_\mathrm{N} + \text{Mass consumed}_\mathrm{S}}$$

Experiments 2bi and 2bii (PER conditioning)

In August 2020 (experiment 2bi) and July 2023 (experiment 2bii), *Apis mellifera carnica* foragers were captured in the same way as in the preference assay (experiment 2a).

Pre-exposure phase

Again, as in experiment 2a, bees were pre-exposed for 72 h to either 1 M sucrose or 1 M sucrose containing 25 µM nicotine.

Harnessing, feeding, and selection of subjects

After 72 h, each bee was removed from its box, chilled on ice, and restrained inside a harness. One hour later, bees were fed to satiation with the original pre-exposure solution (experiment 2bi, 'chronic' treatment) or 1 M sucrose solution (experiment 2bii, 'intermittent'; Fig. 2, column 4). Bees were then left overnight inside a box on the bench, humidified using tissue paper soaked in deionised water (experiment 2bi) or in a climate chamber at 34°C, 60% RH (experiment 2bii). Motivated individuals were selected 19 - 23 h after feeding by applying a droplet of 0.7 M sucrose to the antennae. Only bees with an intact proboscis extension reflex (PER) were retained for the experiment.

Training phase

Each bee was placed into the conditioning arena and randomly assigned a blinded reward solution – either 0.7 M sucrose or 25 µM nicotine in 0.7 M sucrose. Subjects were given 6 conditioning trials, each separated by a 30 s intertrial interval. At each trial, bees were presented with an odour pulse of 1-hexanol for 4 seconds, and then immediately fed with 0.4 µL of reward. A positive result was scored if bees fully extended their proboscis to the odour alone. Bees were removed from the experiment if they did not consume the entire droplet of reward solution on any of the six conditioning trials, or if they displayed a PER to the first odour presentation.

Memory tests

Ten minutes after each bee was trained, her short-term memory (STM) of the odour was scored in a non-reinforced test trial. One hour after conditioning, bees were then fed to satiation with 1 M sucrose (both sub-experiments, Fig. 2) and left overnight. A second unreinforced test trial was performed at 24 h after training to assay the bee's long-term memory (LTM). Data collection continued until results had been obtained from approximately 30 surviving individuals per treatment group per experimental subset.

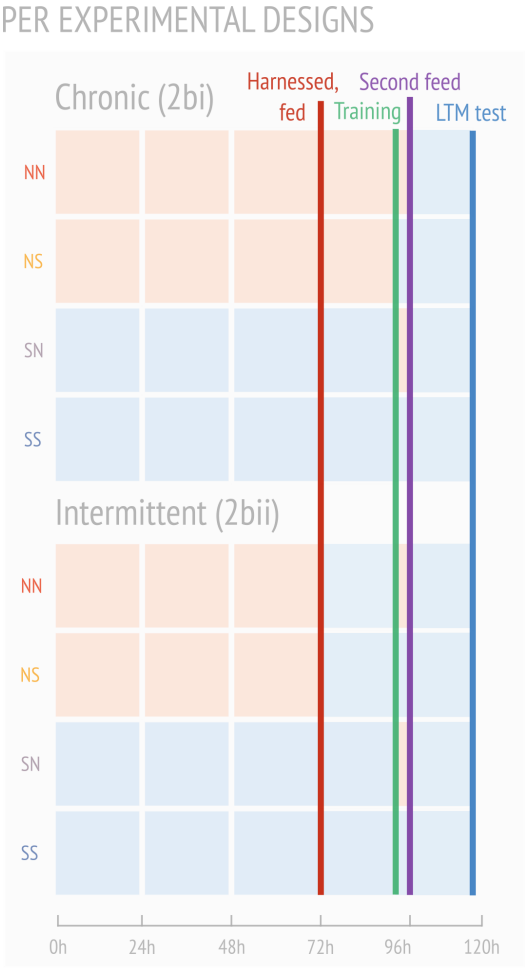


Figure 2 | Schematic diagram of the PER experimental designs, highlighting which solution bees in each treatment group were experiencing. Time in experiment is labelled on the bottom axis. Shaded shapes denote which solution bees had been fed: the colour (orange or blue) denotes the solution bees received (nicotine-laced sucrose or pure sucrose, respectively), and the width indicates the duration of this period, with squares representing 24 h.

Data analysis

All analysis was performed in R version 4.1.2 (R Core Team 2021). Graphs were made using ggplot2, and panels produced in inkscape.

Caged assay (experiment 2a)

Preference index values were bounded between -1 and 1 but approximately normally distributed and concentrated in the middle of this range with no extreme values. Variance was found to be different per group, as per `leveneTest(PI ~ Pre-exp)`. A linear model was fitted that tested for an interaction between pre-treatment group (naive, pre-exposed no WD, or pre-exposed WD) and test type (no choice or choice) (`lm(PI ~ Pre-exp*Test`). Model fit was verified visually using diagnostic graphs. Parameter significance was then tested with the `anova()` function, and heteroscedasticity-robust standard errors (`HC3`) were used to calculate the estimated marginal means when performing the planned comparisons in order to obtain estimates that were adjusted for the unequal variances, i.e. `emm <- emmeans(m, Pre-exp * Test, vcov. = vcovHC(m, type = "HC3"))`. All treatments were compared with 0 (`test(emm, null = 0)`), all within-treatment comparisons were conducted (`pairs(emmeans(m, Test | Pre-exp, vcov. = vcovHC(m, type = "HC3"))`), and the three nicotine choice groups were compared against one another (`contrast(emm_X, method = list("NaiveX vs Pre-expX" = c( 1, -1, 0), "NaiveX vs WDX" = c(1, 0, -1), "Pre-expX vs WDX" = c( 0, 1, -1), adjust = "holm")`.

PER: general statistical methods

Initial models of variables studied in the PER experiment tested for interactions of biological interest and were refined via stepwise selection. Predictor significance was tested using the `Anova()` function in the `car` package (Fox & Weisberg 2019). Parameter estimates and significance for starting vs refined models are viewable in the supplementary tables alongside the model formulae. Graphs were made using `ggplot2`.

PER: survival and acquisition probability

Bees did not all survive to the LTM test. We first analysed survival probability for biological interest and because we had to identify whether to retain or filter out these individuals in our analysis of treatment effects on learning and memory. We also analysed the probability that individuals in each treatment group would acquire the association i.e. respond positively in ≥ 1 trial. For each response in each experiment, we made logistic regression models with the `glm()` function with a binomial error structure and a logit link that first tested for an interaction (*proportion* $\sim$ *pre-exp* $\times$ *reward*).

PER: learning and memory

To estimate population-level effects across trials 2-6 of the learning data (all bees were naive to the odour at trial 1) and across the retention tests (trial 6, STM, LTM) while accounting for repeated measures, we made GEE models using the `geeglm()` function from the `geepack` package (Halekoh et al. 2006). These used an `ar1` correlation structure and individual bee ID as the grouping factor. Learning data from each experiment were tested for whether drugged rewards modified the rate of learning, and whether such an effect could depend on pre-exposure type (*pre-exp* $\times$ *reward* $\times$ *trial*). Memory data were analysed in a similar way (*pre-exp* $\times$ *reward* $\times$ *test*).

Where relevant, pairwise comparisons were performed using `emmeans` (Lenth 2023). To investigate drug withdrawal, we also set out to test for behavioural differences between bees in the NN and SS groups vs NS bees. Where select comparisons were made, we used a Sidak correction. Otherwise, all combinations were Bonferroni-corrected.

RESULTS

Experiment 2a (Choice assay)

Bees responded differently overall in the tests depending on their pre-treatment type ($F_{158} = 9.04, p < 0.001$; Fig. 3; Table S1) and whether given the choice to feed on nicotine-laced food in the test ($F_{158} = 5.54, p = 0.020$; Fig. 3; Table S1). The interaction between the two was marginal ($F_{158} = 2.35, p = 0.099$; Fig. 3; Table S1). On closer inspection, bees in the nicotine withdrawal treatment expressed a significant preference for the drug (WDX vs 0, $p < 0.001$; Table 2) whereas bees in the 3-day exposure group did not (Pre-expX vs 0, $p = 0.14$; Table 2); and nicotine preference was significantly higher in the withdrawal group than following either of the other exposure types (Pre-expX vs WDX: $p = 0.034$; NaiveX vs WDX: $p < 0.001$; Table 2).

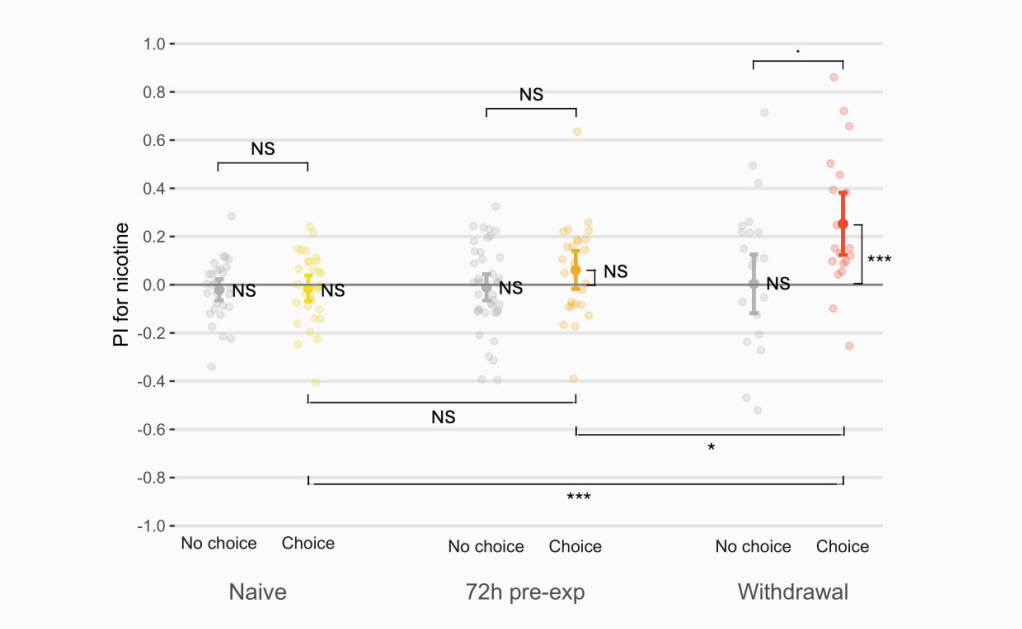


Figure 3 | **Withdrawal induced stronger preferences for nicotine in experiment 2a**. Bees on the left were naive to the drug. Those in the middle had been pre-exposed to it for 72h before the two-way choice, and bees on the right received the same 72 h pre-exposure and were then withdrawn from nicotine-laced food and fed 1M sucrose alone for an additional 24h before the choice phase. Groups illustrated in grey received non-drugged sucrose in both feeders, whereas those in colour were given a choice between nicotine-laced sucrose and non-drugged food. The PIs represent the preference for a random focal sucrose feeder or for nicotine, respectively. n: Naive0 = 30, NaiveX = 30, Pre-exp0 = 40, Pre-expX = 25, WD0 = 20, WDX = 19.

Table 2 | Tukey-adjusted planned *post hoc* pairwise comparisons of group preference indices

Treatment vs 0	Est.	SE	*t*-ratio	*p*
Naive0 – 0	-0.021	0.023	-0.916	0.361
NaiveX – 0	-0.014	0.027	-0.524	0.601
Pre-exp0 – 0	-0.011	0.028	-0.381	0.704
Pre-expX – 0	0.061	0.041	1.49	0.140
WD0 – 0	0.064	0.072	0.889	0.375
WDX – 0	0.253	0.068	3.73	< 0.001 ***
Within group				
Naive0 – NaiveX	-0.007	0.036	-0.188	0.851
Pre-exp0 – Pre-expX	-0.072	0.050	-1.44	0.152
WD0 – WDX	-0.189	0.099	-1.91	0.057 .
Between choice treatments				
NaiveX vs Pre-expX	-0.076	0.050	-1.53	0.129
NaiveX vs WDX	-0.267	0.073	-3.66	0.001 ***
Pre-expX vs WDX	-0.191	0.079	-2.41	0.034 *

Experiments 2bi and 2bii (PER)

Survival

In the 'chronic' experiment, 2bi, survival was marginally lower following nicotine pre-exposure (*pre-exposure*: $\chi^2_1 = 3.37$, $p = 0.066$, Table 3; Fig. 4A, left). Conditioned reward type did not influence survival, nor did it modify the survival outcomes of bees in either pre-exposure treatment (Table 3). We also examined the *post hoc* pairwise contrasts in survival rates between in each group to assess whether survivorship of bees in the withdrawal ('NS') treatment was lower. It was on average, but not significantly (Fig. 4A, left; Table S2a).

In the 'intermittent' experiment (2bii), pre-exposure had no bearing on survival to LTM (Fig. 4A, right; Table 3). We again found that survival rates were lower on average but not significantly for the nicotine-deprived bees (Table S2b).

To facilitate balanced comparisons across the retention tests, we decided to focus further analyses on the bees that survived the full duration of the experiments.

Table 3 | Logistic regression for survival data. Greyed out interactions had been removed prior to estimating final model parameters

Experiment 2bi	χ^2	df	*p*
Pre-exposure	3.37	1	0.066 .
Reward	0.071	1	0.790
Pre-exposure * reward	0.837	1	0.360
Experiment 2bii	χ^2	df	*p*
Pre-exposure	0.076	1	0.783
Reward	1.08	1	0.299
Pre-exposure * reward	2.71	1	0.100

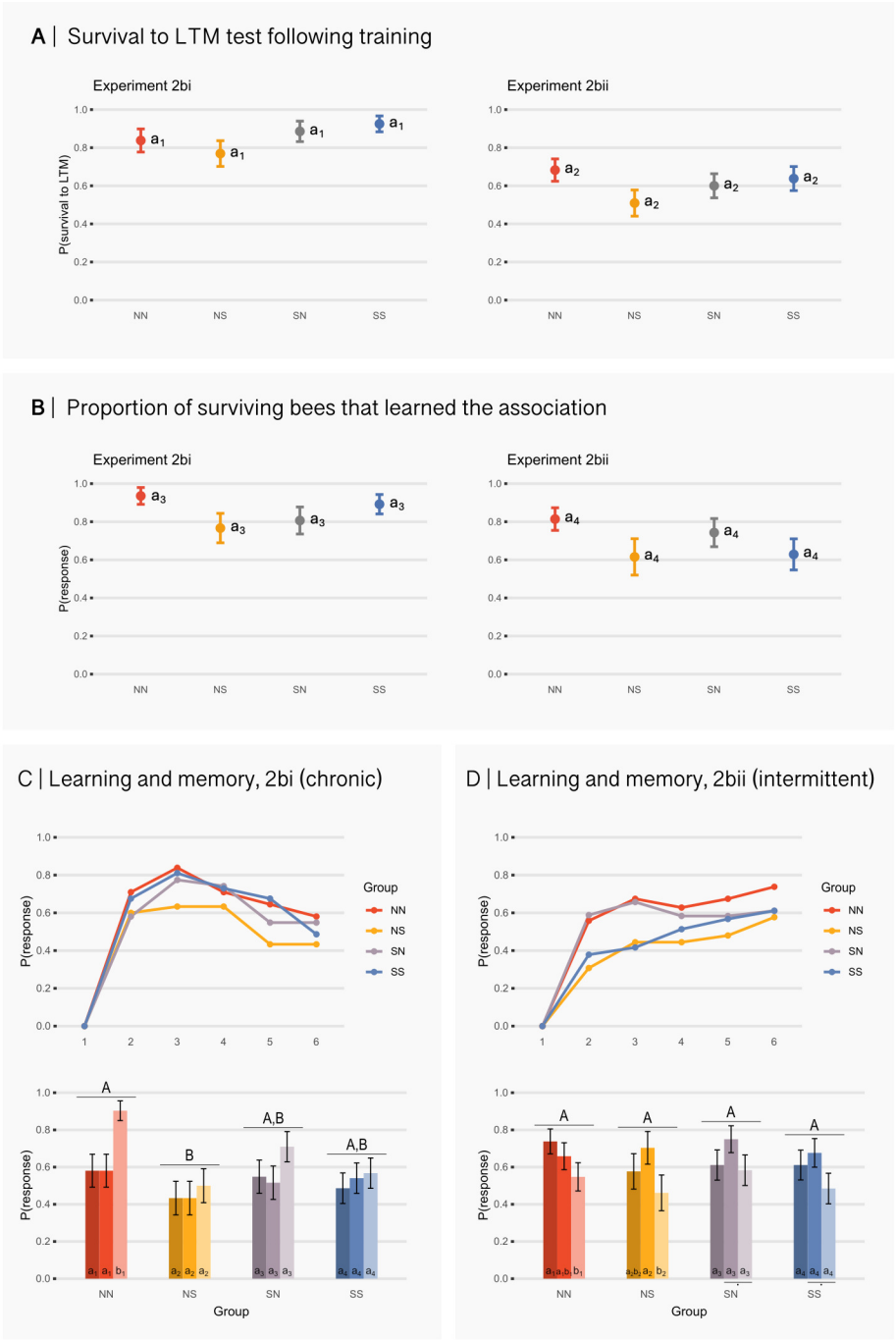


Figure 4 | **Results from experiments 2bi and 2bii. (A) Probability of survival to the LTM test by pre-exposure and conditioned reward solution type**. Most bees survived the experimental duration, though slightly more on average in experiment 2bi and consistently fewest in the NS treatment. Points show mean rate and bars 95% binomial CIs. (n: NN_{2i} = 31/37, NS_{2i} = 30/39, SN_{2i} = 31/35, SS_{2i} = 37/40; NN_{2ii} = 43/63, NS_{2ii} = 27/53, SN_{2ii} = 36/60, SS_{2ii} = 37/58). **(B) Proportion of surviving bees responding with a positive response in $\geq$1 trial by experiment and treatment group**. Most of the bees that survived went on to acquire the association, though the rates of doing so were lowest on average for bees in the NS group (n: NN_{2i} = 29/31, NS_{2i} = 23/30, SN_{2i} = 25/31, SS_{2i} = 33/37; NN_{2ii} = 35/43, NS_{2ii} = 16/26, SN_{2ii} = 26/35, SS_{2ii} = 22/35). **(C) The rate and extent of learning of surviving bees by group in experiment 2bi (withdrawal at LTM), and the probability that they expressed conditioned responding at trial 6 (bottom, darkest bars) vs STM (middle bars) vs LTM (lightest bars)**. Around half were expressing the conditioned response by trial 6 and little change was observed 10 minutes later, but bees trained with nicotine in reward responded more in the LTM tests. Bars show the proportion of subjects in the group demonstrating at least one positive response across the conditioning period ± binomial SE. n: NN = 31, NS = 30, SN = 31, SS = 37. Capital letters above bars denote significance of overall treatment group differences; lower case letters at the base of bars indicate within-group differences in response rates between the tests. **(D) Learning and memory of bees in the second cohort (experiment 2bii: withdrawal at conditioning)**. Baseline learning and memory dynamics were different in this experiment, but bees in the NS treatment tended again to be less responsive during learning than those in the NN group. Trends in the memory data were less clear. n: NN = 43, NS = 27, SN = 36, SS = 37).

Acquisition

In experiment 2bi, bees from either pre-exposure group responded differently to the conditioned rewards (*pre-exposure* × *reward*: $\chi^2_1 = 4.28$, $p = 0.039$; Table 4; Fig. 4B, left) such that bees potentially acquired the association less frequently when deprived of nicotine during conditioning ($p = 0.080$; Tables S3a, S3b) whereas reward type did not influence acquisition probability in naive bees (Table S3a).

In experiment 2bii, bees were instead more likely overall to respond in one or more conditioning trials when rewarded with nicotine, regardless of pre-exposure type (*reward*: $\chi^2_1 = 3.93$, $p = 0.047$, Table 4; Fig. 4B, right; Table S3c).

Table 4 | Logistic regression for acquisition data

Experiment 2bi	χ^2	df	*p*
Pre-exposure	0.00	1	0.970
Reward	0.320	1	0.572
Pre-exposure * reward	4.28	1	0.039 *
Experiment 2bii	χ^2	df	*p*
Pre-exposure	0.203	1	0.652
Reward	3.93	1	0.047 *
Pre-exposure * reward	0.377	1	0.539

Learning and memory, experiment 2bi

In the learning phase of experiment 2bi, conditioned responding declined on average across trials 2-6 (Fig. 4C, top; *trial*: $\chi^2_1 = 8.42$, $p = 0.004$, Table 5). No main treatment effects were detected, nor any significant interactions between treatment variables and trial number (Table S4a). Bees in the NS group consistently expressed the PER least often (Fig. 4C, top), but not significantly less than those pre-exposed to and rewarded with the drug (Table S4b).

General responsiveness across trial 6 and the memory tests was not influenced by pre-treatment (Fig. 4C, bottom; Table 5) but increased when bees were trained with nicotine-laced reward (*reward*: $\chi^2_1 = 4.91$, $p = 0.027$, Table 5), independent of previous exposure (Table S4c). Bees were more likely to respond at the LTM testing point than in the previous tests (*test*: $\chi^2_2 = 8.49$, $p = 0.014$, Table 5), but it is possible that this effect was driven by a boost in responding at LTM experienced only by the nicotine-trained bees (*reward* × *test*: $\chi^2_2 = 5.49$, $p = 0.064$; Tables 5 and S4d).

Table 5 | Parameter significance in reduced GEE models for the learning and memory phases of experiment 2bi

Learning	χ^2	df	*p*
Pre-exposure	0.08	1	0.783
Reward	0.65	1	0.419
Trial	8.42	1	0.004 **
Memory	χ^2	df	*p*
Pre-exposure	0.03	1	0.867
Reward	4.91	1	0.027 *
Test	8.49	2	0.014 *
Reward * test	5.49	2	0.064 .

Learning and memory, experiment 2bii

Bees in experiment 2bii increased their rates of responding over trials 2-6 (*trial*: $\chi^2_1 = 19.0$, $p < 0.001$; Table 6, Fig. 4D, top). Conditioned responding was higher on average across the training period in experiment 2bii when bees were trained on nicotine than sucrose (*reward*: $\chi^2_1 = 4.50$, $p = 0.034$; Table 6) though the rate of learning was not significantly faster (*reward* × *trial*: $p = 0.112$, Table S5a). Pre-exposure type made no difference overall to learning success (Table 6), and did not modify the influence of either main effect (Table S5a).

In experiment 2bii, rates of responding across T6 and in the memory tests varied (*test*: $\chi^2_2 = 15.4$, $p < 0.001$; Table 6), but no treatment effects or interactions were detected (Table S5c). Unlike in the previous experiment (2bi), bees did not respond significantly more in the memory tests when rewarded with nicotine (*reward*: $\chi^2_1 = 1.49$, $p = 0.223$; Table 6).

Table 6 | Parameter significance in reduced GEE models for the learning and memory phases of experiment 2bii

Learning	χ^2	df	*p*
Pre-exposure	0.16	1	0.686
Reward	4.50	1	0.034 *
Trial	19.0	1	< 0.001 ***
Memory	χ^2	df	*p*
Pre-exp	0.06	1	0.812
Reward	1.49	1	0.223
Test	15.4	2	< 0.001 ***

DISCUSSION

We performed a series of experiments to test the mechanisms by which exposure to nicotine in food influences reward valuation and feeding behaviour in honeybees. We first showed that extended exposure to nectar-relevant levels of nicotine in food can induce robust preferences for food laced with the drug after a period of abstinence. We then performed 6-trial massed olfactory conditioning using nicotine-laced food to investigate its effect on reward valuation in naive bees more closely, and to seek further evidence of nicotine addiction in a different experimental context. We did not find clear evidence of an improvement to the learning or memory of drug-naive bees with nicotine-laced reward, but nicotine pre-exposed bees showed slight yet consistent differences in their responses to the two reward types in ways consistent with the hypothesis of addiction.

Bees in the choice assay showed withdrawal-dependent preferences for nicotine

As a nAChR agonist, nicotine could hypothetically function as a positive reinforcer to improve the perceived quality of a food source. In our caged assay, we found that preference was predicted by the presence of a choice, such that, overall, nicotine-laced food was more likely to be consumed. However, the only bees that clearly favoured this food source were those in the withdrawal group, and naive bees did not show any discernable bias towards the drug. This appears to suggest either that they were indifferent to it, unable to detect it, or unable to learn to distinguish the feeders using spatial information alone.

An assumption made with this method is that individuals will consume more of a preferred food source. This seems reasonable for addictive studies given that the early stages of drug abuse are typically marked by patterns of binging behaviour driven by hedonic reinforcement. However, nicotine addiction is unusual in that intake is usually highly titrated (Koob 2008) and likely to be governed by stringent homeostatic feedback loops. It is arguably also possible, therefore, that this metric was not sensitive enough to capture subtle and transient bouts of behavioural evidence that would indicate that naive bees find the drug rewarding. To do so, it might be helpful to see how naive bees choose to interact with the drug in a two-way choice test over time.

The results from the other choice treatments indicate that bees are capable of telling the feeders apart and biasing their feeding decisions in this context to favour nicotine-laced food. Regardless of whether bees can taste 25μM nicotine in relatively concentrated sugar solutions, which is arguably unlikely – this is a low drug concentration, and honeybees have limited bitter detection – this was probably not due to the development of an acquired taste preference because strong preferential feeding (as measured here) was only instantiated following a period of abstinence. It seems more likely that the different pre-treatment regimes produced differences in how bees valued each food source, but statistical evidence for this was marginal.

We were surprised to observe that the variance in measured preferences scaled with the time bees spent in the cages during the pre-treatment phases. This clearly has the potential to alter choice behaviour, and could potentially therefore have exerted some sort of confounding influence on nicotine responses in the choice tests. With hindsight, this should have been standardised, perhaps by introducing initial sucrose feeding time windows. We therefore attach a note of caution to the following conclusions.

These results seem to demonstrate that repeated exposure to nectar-relevant nicotine levels in food can alter bee behaviour in ways that are likely in flux with an animal's chemical homeostasis. This is consistent with the hypothesis that the animals can become addicted to it, but the evidence from these data is not conclusive.

Motivational states varied in the PER experiments

In the PER experiments we aimed to examine the effect of nicotine on food reward valuation more closely, using analogous pre-exposure treatments to those in the caged assays.

We saw salient differences in the baseline behavioural responses of bees across experiments which somewhat complicates our ability to compare between them. Specifically, bees in experiment 2bi seem to have experienced a ceiling effect on their learning by trial 3 of conditioning, and were generally (and somewhat atypically for these experiments) more motivated to respond at the LTM test than on the previous day. By contrast, bees in experiment 2bii experienced lower rates of survival and acquisition, and showed slower but more sustained improvements to their learning.

Discrepancy in the overall dynamics of learning and memory is not uncommon between PER experiments. In our study, some of this noise may also be explained by protocol differences: bees were left out overnight to LTM testing on the bench in a humid box in experiment 2bi, but placed in a dark climate chamber in experiment 2bii. If the latter is a more stressful environment it may explain the reduced survival rate, and stress can reduce the expectation of food reward in bees during learning tasks (Procenko et al. 2024, Bate-

son et al. 2011). Our analyses only focussed on survivors of the full experiment, which may have gone some way to standardising the levels of stress and motivation to feed of the test subjects. Whatever the causes, it was nonetheless difficult to do so across experimental subtypes and between the testing points.

We highlight this because stress state and motivation to feed might be linked to an individual's susceptibility to the effects of nicotine (George & Koob 2017). Given that bees in groups NN and NS were in a drug-deprived state at different points in the experimental rounds (during the LTM tests in experiment 2bi and conditioning in experiment 2bii), any interpretations we make about the timing of withdrawal by comparison across these datasets are done so with caution.

Nicotine exposure tended to reduce survival

Nicotine intake may affect bee longevity in several ways, so we monitored the survival of bees from training to the LTM test.

Withdrawal from certain addictive substances can be deadly to humans. Nicotine cessation is benign in comparison, but repeated exposure of animal models to the drug nonetheless decreases dopamine release in reward pathways and sensitises stress pathways, which, under withdrawal, can precipitate a disease state (Koob & Schulkin 2019). One study found that bees were more likely to die after extended ethanol exposure when the drug was withheld (Ostap-Chec et al. 2021). We predicted that we might see higher mortality of bees in the NS group, which had been withdrawn from and deprived of the drug during training.

Nicotine may also modulate survival through other mechanisms. Studies probing it for medicinal effects in bees find, in general, that food laced with nicotine or other similar compounds does not modify the survival outcomes of healthy control bees (Richardson et al. 2015, Biller et al. 2015, Palmer-Young et al. 2015) but can help to reduce mortality of infected bees (Köhler et al. 2012a, Baracchi et al. 2015, Manson et al. 2010, Anthony et al. 2015, Palmer-Young et al. 2017b; though see Köhler et al. 2012b, Palmer-Young et al. 2017a). By contrast, when delivered in combination with alternative forms of stress, authors tend to report that it exacerbates forager mortality (Köhler et al. 2012b, Archer et al. 2014). This is perhaps to be expected given nicotine's ability to upregulate stress responses.

We found that survival consistently tended to be lowest on average in the NS group. This perhaps hints at a withdrawal effect that may be mitigated by exposure to nicotine in reward during conditioning, but we don't have the statistical power to claim so with confidence. It may be that mortality rate is not a sufficiently high-resolution metric for capturing these effects.

We did also find that marginally fewer nicotine pre-exposed bees – individuals from both the NS and NN groups – survived to the LTM test in experiment 2bi but not 2bii. Again, this could plausibly be attributed to chance. It seems unlikely that this evidences synergy between the drug and other stressors, because survival rates were generally higher in this round. Instead, it may be due to differential retention of the more stress-prone subjects: bees in these groups in this experiment (withdrawal at LTM) both experienced the onset of a withdrawal period after training, whereas in experiment 2bii (withdrawal during conditioning), bees had already had to endure nearly 24 h of nicotine abstinence in order to be recruited. This result also seems to imply that bees in experiment 2bii were not experiencing greater levels of withdrawal stress, potentially because they were exiting the withdrawal window between training and testing. To confirm, it may be useful for the timescales of nicotine withdrawal in honeybees to be determined.

Nicotine-laced reward may improve the cognition of drug-naive bees, but not in straightforward ways

Bees in the SN group were fed according to the same protocol across experiments 2bi and 2bii.

Acquisition probability appeared similar between these groups in either experiment, but we found inconsistencies in their learning and memory dynamics. In experiment 2bi, they learned no differently to the control (SS) but responded slightly more at the LTM test. Given that the bees in the NN group also experienced a boost to their LTM, it perhaps seems plausible that nicotine can selectively improve consolidation and/or retrieval. Baracchi et al. (2017) found that bees showed more latency to update their behaviour in reversal learning after experiencing nicotine in reward, and this has been interpreted as an indication it can generate more persistent memory traces. However, in experiment 2bii this effect on memory was absent, and bees instead exhibited higher rates of conditioned responding during training with nicotine-laced reward, particularly earlier in the conditioning period. Response rates contain information about many things including cognitive ability, perceived reward quality, and motivational state. Nicotine could conceivably have interacted with any of these factors in complex ways, which perhaps explains these disparities.

The data from experiment 2bi suggest that nicotine does not enhance learning in drug-naive bees. This may be true, but it could be possi-

ble that the response rates in the control were already nearing a biological maximum: on average, responding peaked by trial 3, reaching rates of >80%. A ceiling effect could potentially obscure any pharmacological enhancement to learning. Conditioning bees with rewards of lower sugar concentrations may help to clarify if this is the case. In experiment 2bii, when bees were slower to learn, increased responsiveness to nicotine-laced reward emerged in the first half of the conditioning period. In the forward-learning component of the study by Baracchi et al. (2017), they found that, in a task analogous to ours, nicotine-laced nectar modified the behaviour of free-flying bumblebees more in the earlier learning trials. We expect nicotine to reach the bee brain within about 30 seconds of ingestion (Simcock et al. 2018), and perhaps it may be that nicotine-laced rewards can serve to improve processes with greater involvement in the earlier stages of learning such as attentional focus.

In sum, it seems that nicotine in food may have the potential to modify reward learning and memory in naive bees but these effects are not easily repeatable, potentially due to motivational dependences and disparities.

Did we see evidence of withdrawal?

We hypothesised that bees in a drug-deprived state would acquire the association less frequently due to cognitive deficits (Calu et al. 2007, Barron et al. 2009, Hall et al. 2015). We did not know whether this would be the case if they were trained with nicotine-laced reward (NN) or whether these bees would respond disproportionally well, as they might if they valued the reward more highly.

Rates of acquisition were consistently but non-significantly lowest for bees in the NS group, especially in comparison to the other bees that had been pre-exposed to nicotine and trained on it (NN). However, this was no more true of bees that were hypothetically in withdrawal during the learning task (experiment 2bii). This seems to imply that nicotine pre-exposure could potentially change the way in which bees tend to respond to either solution during a learning task, but, if so, the processes responsible may be less dependent on a chemical homeostatic state than we had expected.

We expected to see somewhat similar learning dynamics between bees in the NN and NS groups in experiment 2bi given that nicotine may still have been present in the hemolymph. This isn't really what we observed: no significant group differences were detected, but NS bees consistently responded least across the trials. This may agree with the choice assay results, in which nicotine pre-exposure without a withdrawal period caused bees to develop a non-significant preference for nicotine; and together perhaps suggests that nicotine pre-exposure could induce a process by which a natural sucrose reward is devalued relative to the nicotine-laced food. Blunted responses to natural rewards are also an established marker of addiction (Volkow et al. 2019, Grigson 2008, McFalls et al. 2016) which, to our knowledge, has not previously been documented in bees.

We didn't know how nicotine pre-exposed bees in experiment 2bi would react at the LTM test once withdrawn. We assumed that their state would have little if any influence on conditioned responding (Kacelnik et al. 2023) and expected to see carryover of response rates. Interestingly, these bees responded much less to the odour at 24 h when it predicted a sucrose reward (NN vs NS), and tended to respond more to the nicotine-associated stimulus following drug pre-exposure (NN vs SN). At first glance it looks as though bees are somehow aware of a drug's presence in reward and are able to retain and act on this information, making use of sophisticated mechanisms for monitoring and maintenance of chemical homeostasis. A more parsimonious explanation may be differences mid to long term memory consolidation by drug pre-exposed bees, perhaps linked to drug sensitisation and reduced natural reward sensitivity.

In experiment 2bii, we expected drug-deprived bees to respond more to nicotine-laced reward and less to sucrose. This time, all bees – but especially those in withdrawal – learned significantly better when rewarded with nicotine. It thus seems able to modify reward learning via a combination of pre-exposure-dependent and independent mechanisms. These differences were not instantiated as expected in the LTM test, and bees in the NN group responded significantly less than on the previous day. It could just be that the withdrawal window had closed by this time point, but that would again pre-suppose the existence of mechanisms in bees for updating preferences according to their state.

Conclusions

Across our assays, we found that 72 h of forced exposure to nectar-relevant nicotine levels in food produced several changes in behaviour indicative of addiction, though these findings are not conclusive. In the preference experiment bees began to value nicotine more relative to sucrose, and this resulted in a robust preference when bees had been drug-deprived for 24 h prior to testing. In the PER experiments, we found that bees were

in quite different motivational states which were possibly linked to stress. Despite this, we found subtle but consistent evidence that bees did behave differently under withdrawal in ways that suggested that they were devaluing natural rewards and possibly had been sensitised to the effects of nicotine in food. Evidence for cognitive enhancement in naive bees was more equivocal.

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Data availability

The data obtained from all experiments in this chapter are available to download on GitHub, as are the R files used to visualise and analyse them, at https://github.com/annadorling/Nicotine-preferences.

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SUPPLEMENTARY INFORMATION

1 Caged assay

Table S1 | Parameter significance in linear model for caged assay

	df	Sum sq.	Mean sq.	F	*p*
Pre-exposure	2	0.756	0.378	9.04	< 0.001 ***
Test	1	0.232	0.232	5.54	0.020 *
Pre-exposure * Test	2	0.196	0.098	2.35	0.099 .
Residuals	158	6.61	0.042		

2 Survival data

Post hoc tests of group differences in survival by experiment

Table S2a | Tukey-adjusted *post hoc* pairwise comparisons for survival data in experiment 2bi

Contrast	Estimate	SE	df	z-ratio	p-value
NS - SS	-1.31	0.710	Inf	-1.84	0.254
SN - SS	-0.465	0.802	Inf	-0.580	0.938
SN - NS	0.844	0.653	Inf	1.29	0.568
NN - SS	-0.870	0.748	Inf	-1.16	0.650
NN - NS	0.438	0.586	Inf	0.748	0.878
NN - SN	-0.405	0.694	Inf	-0.585	0.937

Table S2b | Tukey-adjusted *post hoc* pairwise comparisons for survival data in experiment 2bii

Contrast	Estimate	SE	df	z-ratio	p-value
NS - SS	-0.529	0.387	Inf	-1.36	0.522
SN - SS	-0.161	0.380	Inf	-0.424	0.974
SN - NS	0.368	0.381	Inf	0.966	0.769
NN - SS	0.199	0.385	Inf	0.518	0.955
NN - NS	0.728	0.386	Inf	1.89	0.234
NN - SN	0.360	0.378	Inf	0.953	0.776

3 Acquisition data

Post hoc tests of group differences in acquisition within each experiment

Table S3a | Pairwise comparisons of reward type within each pre-exposure condition to investigate the pre-exposure*reward interaction

Pre-exp	Reward contrast	Estimate	SE	df	z-ratio	p-value
S	N - S	-0.683	0.698	Inf	-0.979	0.328
N	N - S	1.49	0.849	Inf	1.75	0.080 .

Table S3b | Tukey-adjusted *post hoc* pairwise comparisons for acquisition data in experiment 2bi

Contrast	Estimate	SE	df	z-ratio	p-value
NS - SS	-0.921	0.683	Inf	-1.35	0.532
SN - SS	-0.683	0.698	Inf	-0.979	0.762
SN - NS	0.238	0.627	Inf	0.379	0.982
NN - SS	0.564	0.903	Inf	0.625	0.924
NN - NS	1.49	0.849	Inf	1.75	0.299
NN - SN	1.25	0.861	Inf	1.45	0.469

Table S3c | Tukey-adjusted *post hoc* pairwise comparisons for acquisition data in experiment 2bii

Contrast	Estimate	SE	df	z-ratio	p-value
NS - SS	-0.056	0.534	Inf	-0.105	1.00
SN - SS	0.535	0.521	Inf	1.03	0.734
SN - NS	0.591	0.559	Inf	1.06	0.715
NN - SS	0.950	0.525	Inf	1.81	0.269
NN - NS	1.01	0.562	Inf	1.79	0.278
NN - SN	0.415	0.551	Inf	0.754	0.875

4 Learning and memory, experiment 2bi

Learning

Table S4a | Type III Wald chi-square tests for the initial vs simplified GEE models of learning in experiment 2bi

	Initial model			Simplified model		
Predictor	χ^2	df	p-value	χ^2	df	p-value
Pre-exposure	0.08	1	0.783	0.08	1	0.783
Reward	0.65	1	0.419	0.65	1	0.419
Trial	8.42	1	0.004 **	8.42	1	0.004 **
Pre-exposure * Reward	1.77	1	0.184			
Pre-exposure * Trial	0.08	1	0.774			
Reward * Trial	0.75	1	0.387			
Pre-exposure * Reward * Trial	0.49	1	0.483			

Table S4b | Tukey-adjusted *post hoc* group pairwise comparisons for learning data in experiment 2bi

Contrast	Estimate	SE	df	z-ratio	p-value
NN–SN	0.311	0.387	Inf	0.804	0.853
NN–NS	0.609	0.374	Inf	1.63	0.363
NN–SS	0.158	0.333	Inf	0.476	0.964
SN–NS	0.298	0.431	Inf	0.691	0.901
SN–SS	-0.153	0.395	Inf	-0.387	0.980
NS–SS	-0.450	0.382	Inf	-1.18	0.641

Memory

Table S4c | Type III Wald chi-square tests for the initial vs simplified GEE models of memory in experiment 2bi

	Full model			Simplified model		
Predictor	χ^2	df	p-value	χ^2	df	p-value
Pre-exposure	0.01	1	0.904	0.03	1	0.867
Reward	4.45	1	0.035 *	4.91	1	0.027 *
Test	7.49	2	0.024 *	8.49	2	0.014 *
Pre-exposure * Reward	2.30	1	0.130			
Pre-exposure * Test	1.00	2	0.608			
Reward * Test	5.05	2	0.080 .	5.49	2	0.064 .
Pre-exposure * Reward * Test	1.42	2	0.491			

Table S4d | Tukey-adjusted *post hoc* pairwise comparisons of performance at different tests, by reward type

Reward = S				
Contrast	Estimate	SE	*z*-ratio	*p*
T6–STM	-0.160	0.401	-0.400	0.916
T6–LTM	-0.401	0.402	-1.00	0.579
STM–LTM	-0.241	0.401	-0.600	0.820
Reward = N				
T6–STM	0.091	0.427	0.210	0.975
T6–LTM	-1.57	0.493	-3.18	0.004 **
STM–LTM	-1.66	0.495	-3.35	0.002 **

Table S4e | Tukey-adjusted *post hoc* group pairwise comparisons for memory data in experiment 2bi (used for plotting)

Contrast	Estimate	SE	df	z-ratio	p-value
NN–SN	0.737	0.517	Inf	1.43	0.483
NN–NS	1.50	0.526	Inf	2.85	0.023 *
NN–SS	1.09	0.496	Inf	2.20	0.122
SN–NS	0.761	0.490	Inf	1.55	0.405
SN–SS	0.356	0.462	Inf	0.771	0.867
NS–SS	-0.405	0.459	Inf	-0.882	0.814

Table S4f | Tukey-adjusted *post hoc* within-group pairwise comparisons of performance at different tests

Group = NN				
Contrast	Estimate	SE	*z*-ratio	*p*
T6–STM	-0.002	0.606	-0.003	1.00
T6–LTM	-2.42	0.806	-3.00	0.008 **
STM–LTM	-2.42	0.806	-3.00	0.008 **
Group = SN				
T6–STM	0.177	0.603	0.293	0.954
T6–LTM	-0.964	0.635	-1.52	0.282
STM–LTM	-1.14	0.637	-1.79	0.173
Group = NS				
T6–STM	0.006	0.606	0.010	1.00
T6–LTM	-0.362	0.605	-0.598	0.821
STM–LTM	-0.368	0.605	-0.608	0.816
Group = SS				
T6–STM	-0.288	0.536	-0.538	0.853
T6–LTM	-0.434	0.538	-0.807	0.699
STM–LTM	-0.146	0.538	-0.271	0.960

5 Learning and memory, experiment 2bii

Learning

Table S5a | Type III Wald chi-square tests for the full and simplified GEE models

	Full model			Simplified model		
Predictor	χ^2	df	p-value	χ^2	df	p-value
Pre-exposure	0.16	1	0.686	0.16	1	0.686
Reward	4.50	1	0.034 *	4.50	1	0.034 *
Trial	19.0	1	$<$ 0.001 ***	19.0	1	$<$ 0.001 ***
Pre-exposure * reward	0.40	1	0.528			
Pre-exposure * trial	1.34	1	0.247			
Reward * trial	2.52	1	0.112			
Pre-exposure * reward * trial	1.17	1	0.279			

Table S5b | Tukey-adjusted *post hoc* group pairwise comparisons for learning data in experiment 2bii

Contrast	Estimate	SE	df	z-ratio	p-value
NN–SN	0.249	0.409	Inf	0.608	0.930
NN–NS	0.868	0.442	Inf	1.96	0.202
NN–SS	0.689	0.406	Inf	1.70	0.326
SN–NS	0.620	0.456	Inf	1.36	0.525
SN–SS	0.440	0.421	Inf	1.05	0.722
NS–SS	-0.180	0.453	Inf	-0.397	0.979

Memory

Table S5c | Type III Wald chi-square tests for the initial vs simplified GEE models of memory in experiment 2bii

	Full model			Simplified model		
Predictor	χ^2	df	p-value	χ^2	df	p-value
Pre-exposure	0.04	1	0.848	0.06	1	0.812
Reward	1.17	1	0.280	1.49	1	0.223
Test	13.3	2	0.001 **	15.4	2	$<$ 0.001 ***
Pre-exposure * Reward	0.00	1	0.979			
Pre-exposure * Test	1.51	2	0.470			
Reward * Test	0.66	2	0.721			
Pre-exposure * Reward * Test	4.36	2	0.113			

Table S5d | Tukey-adjusted *post hoc* group pairwise comparisons for memory data in experiment 2bi (used for plotting)

Contrast	Estimate	SE	df	z-ratio	p-value
NN–SN	-0.206	0.885	Inf	-0.233	0.996
NN–NS	0.741	0.929	Inf	0.797	0.856
NN–SS	0.637	0.872	Inf	0.730	0.885
SN–NS	0.947	0.988	Inf	0.958	0.773
SN–SS	0.843	0.932	Inf	0.904	0.803
NS–SS	-0.104	0.956	Inf	-0.109	1.00

Table S5e | Tukey-adjusted *post hoc* within-group pairwise comparisons of performance at different tests

Group = NN				
Contrast	Estimate	SE	*z*-ratio	*p*
T6–STM	0.969	0.716	1.35	0.365
T6–LTM	1.85	0.723	2.56	0.028 *
STM–LTM	0.882	0.667	1.32	0.383
Group = SN				
T6–STM	-1.59	0.838	-1.90	0.138
T6–LTM	0.245	0.735	0.333	0.941
STM–LTM	1.84	0.845	2.18	0.075 .
Group = NS				
T6–STM	-1.15	0.837	-1.37	0.356
T6–LTM	0.868	0.819	1.06	0.539
STM–LTM	2.02	0.860	2.35	0.050 *
Group = SS				
T6–STM	-0.429	0.772	-0.556	0.844
T6–LTM	1.35	0.778	1.73	0.193
STM–LTM	1.78	0.810	2.19	0.072 .

Chapter 3 | A method for automated monitoring of feeding choice behaviour in bumblebees

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Abstract

Bees encounter nutrients and non-nutrient compounds in floral nectar. To understand the mechanisms governing the acquisition and use of floral resources – such as taste, learning and memory, and the maintenance of nutritional homeostasis – it is essential to study how nutrients and other compounds influence feeding decisions. Existing experimental paradigms to do so include olfactory conditioning of the proboscis extension reflex, caged assays, free-flight experiments, and manual behavioural scoring-based techniques. These approaches have all been productive but in isolation do not simultaneously allow for extended, high-throughput data capture at fine temporal and behavioural scales. Here, we present a novel, computer vision-based method developed for measuring the choices and the structure of feeding of individual worker bumblebees in response to a set of two choice solutions across unbounded timescales. To verify the assay, we evaluated the deterrent activity of quinine, a known feeding deterrent stimulus to bumblebees, using a variety of behavioural metrics. We also investigated the way that a learned aversion develops by studying the changes in feeding behaviour over time towards quinine. This workflow yields detail-rich datasets for studying the dynamics of feeding behaviour in bees under a two-way choice, with potential applications to a broad set of research questions.

INTRODUCTION

Adaptive behaviour depends on efficient decision making (Emlen 1966). To make decisions, animals have to weigh up the costs and benefits of alternative responses (Dill et al. 1987, Lima & Dill 1990). Their behaviour therefore contains information about their motivational priorities.

Binary food choice assays are a commonly used behavioural test of an animal's motivation to access a particular nutritional resource. In these experiments, animals are presented with two food options and their interactions are quantified in various ways (e.g., the amount of time spent at the feeder, number of bouts, volume change of feeder, latency of approach, duration of first bout, etc.). From the difference in responding between food sources, the relative valence of each can be determined. This can be expressed as a 'preference' for or against a focal food source, and is assumed to be a quantitative reflection of the animal's motivation to obtain it relative to the other option (Bateson 2004).

Neuroactive nectar has been described as an evolutionary paradox (Mustard 2020, Stevenson 2020). We are interested to understand whether putatively addictive compounds in nectar increase the motivation of bees to feed from these food sources. Binary choice assays using equivalent sugar solutions, one of which is laced with the drug, may reveal information about whether drugs in nectar enhance its perceived value.

In insects, binary feeding assays typically quantify the ingested volume of each food source over a defined time period, either (as in chapter 2) by measuring volume change in the feeders (Ja et al. 2007, Kessler et al. 2015) or from the bodies of animals using radiolabel- and colorimetric dye-based methods (Sang et al. 2021, Thompson & Reeder 1987, Deshpande et al. 2014, Aryal et al. 2022). The latter techniques are not optimised for bees. Additionally, although relatively easy and widely used, neither protocol can produce time series data. Responses in these assays are also analysed at a population level, making it hard to explore individual variation.

We expect motivation to self-administer addictive drugs to vary with previous experience and homeostatic state, and therefore to be in flux throughout the experimental duration. It could potentially vary in non-random ways between individuals, too. Existing methods for use in bees cannot capture continuous information about the voluntary interactions individual bees make with either of two test solutions, nor finer-scale behavioural information such as visitation rates to each feeder or the microstructure of ingestion events.

Techniques have been developed that can allow the *ad lib* feeding decisions of individual, freely-moving *Drosophila* to be tracked through time using electrical capacitance measurements (Itskov et al. 2014, Ro et al. 2014) to yield information at fine behavioural and temporal scales. The availability of open-source machine learning-based pose estimation software such as DeepLabCut (DLC) has recently allowed biologists to create computer vision-based methods, too (Mathis et al. 2018, Charroux & Royet 2020, Colombi et al.

2025). Both setups can provide high-throughput analyses that are free of observer bias and respond to the demand for methods that promote reproducible research.

I designed and built a system for automated monitoring of choice feeding behaviour in bees presented with a solution at the end of either arm of a y-maze. Here, I describe the assay and present a pilot study of the behavioural responses of bumblebees to quinine, an alkaloid that is known to be both deterrent and toxic to them (Wright et al. 2010, Hurst et al. 2014, Tiedeken et al. 2014).

First, the rate at which the average bee sampled each food source through time across 1 h time bins was investigated. We monitored the total duration for which individual bees maintained proboscis contact with each, the total number of sips recorded from each, and the number of detected feeding attempts from each food source. We expected that the cumulative quinine-feeding curves would appear shallower and plateau as bees learned to avoid this food source. Cumulative feeding metrics at 6 h (the end of the monitoring period) extracted from the videos were then compared statistically. It was predicted that all measures would be significantly lower when the food was laced with quinine.

Using these behavioural metrics, feeding preferences at 6 h were subsequently indexed for all subjects. These data were used to quantify the magnitude of aversion at the group level using a series of p-adjusted comparisons against 0, and rank-correlated with estimates derived from feeder volume change data to evaluate concordance between methods. Preference index (PI) was also monitored continuously over time at both the individual and population levels using the video-derived behavioural data. At the population level, a stable and moderate level of aversion consistent with an aversive taste response was expected to emerge early on in the monitoring period, detectable visually. The predicted extent of inter-individual variation in PI trajectories and endpoints was uncertain, but some degree of avoidance behaviour was expected to be the modal response.

Additional structural features of feeding behaviour were statistically analysed, including first bout duration, mean bout duration, mean sips per bout, and mean speed of proboscis movement, to evaluate the sensitivity of the assay for detecting behavioural signatures of aversive taste. All measures were predicted to be lower in the presence of quinine than for the pure sucrose solution.

SETUP

Hardware overview

The system comprised a set of 10 behavioural observation modules (Fig. 1a). These each recorded the feeding events of one bumblebee (*Bombus terrestris*).

In each unit, a bee was contained in a white perspex box with a transparent lid. A small Y-maze was attached that was positioned outward towards the test solutions held along the perimeter of the system (Fig. 1b; Fig. S1). The boxes were equidistantly spaced, with rectangular notches along the bottom edge that were slotted into holes on a plastic platform to ensure consistent positioning relative to the cameras.

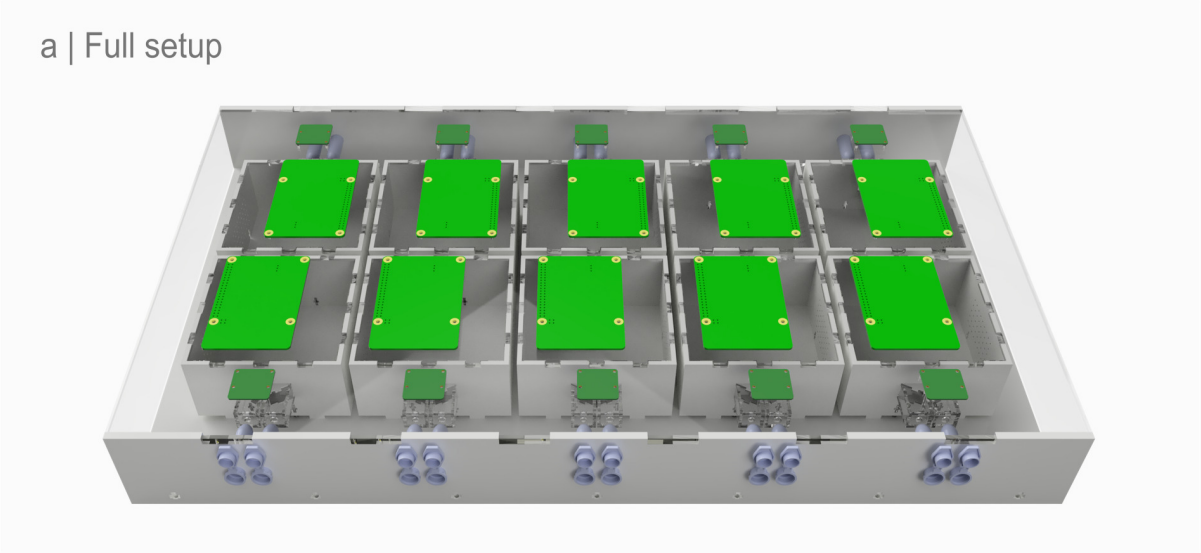
At the end of each arm of the Y-maze was an aperture 2 cm in diameter leading to a 2.5 ml Eppendorf tube, positioned a few mm away, offering food through an opening in the tip. These were inserted through holes in pieces of plastic that enclosed and extended upwards from the platform, perpendicular to its long edges. Once the bees and solutions were in position, with the Y-mazes oriented outward towards the test solutions, a sheet of transparent plastic was slotted into place on top of the arena. This hosted the ten raspberry pis (RPis), camera modules, and breadboards, fixed on with screws, and positioned them above the respective recording area. Each circuit contained five components connected to the GPIOs: four lights were used to provide feedback to the experimenter as to the state of recording, and an input button initiated and terminated the program for data collection (Fig. 1c).

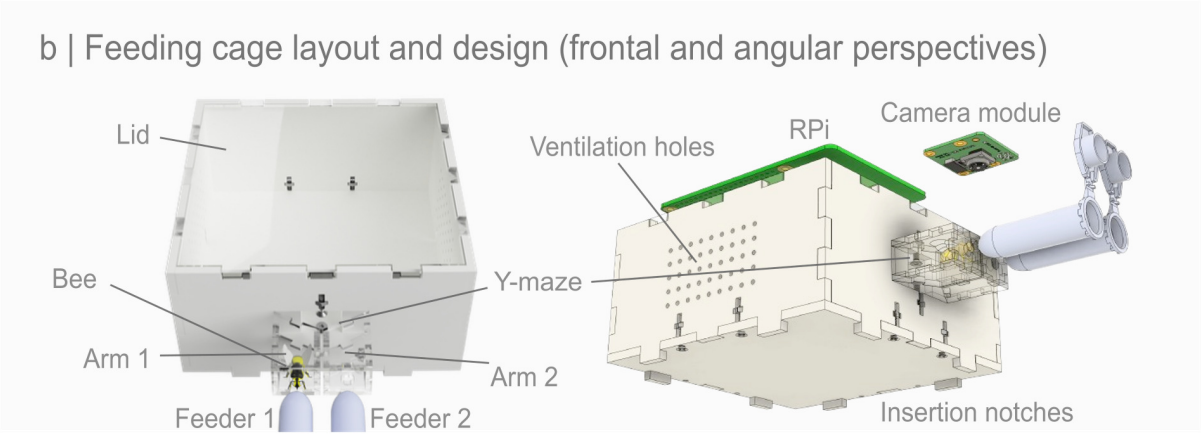
I designed the setup using Fusion 360 CAD software, and laser cut the components of the arena from perspex sheets (Kitronik, Nottingham) using exported `.dxf` files.

Data capture

On boot, the RPis each began running a python script using a `systemd` service file. This program initialised the camera and GPIOs, and waited for input from the button pin. Once pressed, a new directory was created using the time and date, and within it a text file was also initialised to enable logging of the timestamps at which recording events began and ended. On completion, the green LED then flashed several times to mark the beginning of the data collection phase.

The camera module then captured images of the entire choice region at 5fps, and compared each image in sequence to the one before it. Motion between two consecutive images was assessed using the OpenCV (`cv2`) computer vision software library. To do so, images were converted to black and white with `cvtColor()`, filtered





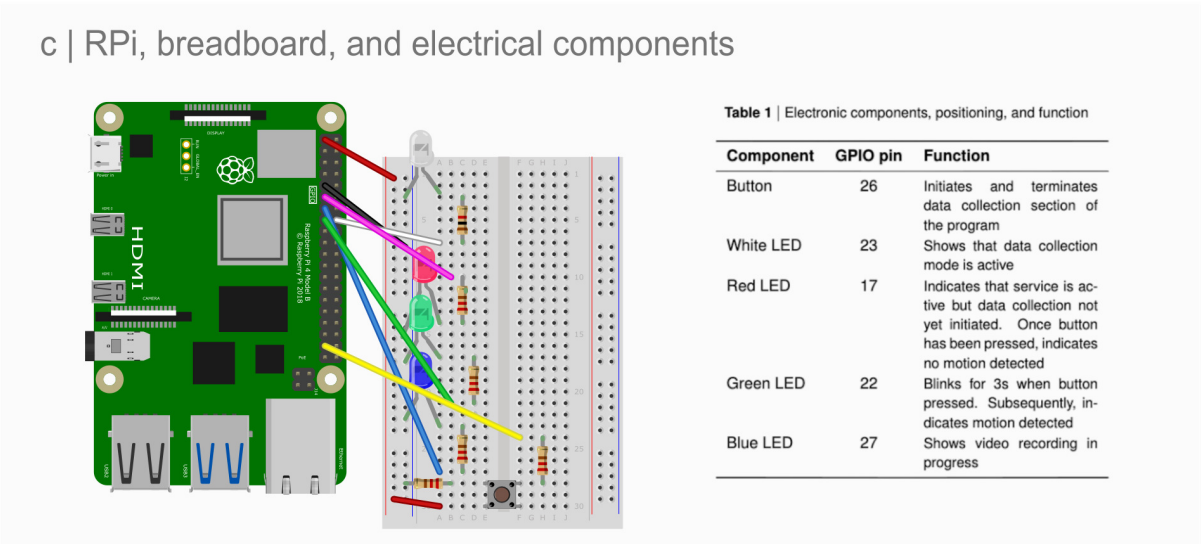


Table 1 | Electronic components, positioning, and function

Component	GPIO pin	Function
Button	26	Initiates and terminates data collection section of the program
White LED	23	Shows that data collection mode is active
Red LED	17	Indicates that service is active but data collection not yet initiated. Once button has been pressed, indicates no motion detected
Green LED	22	Blinks for 3s when button pressed. Subsequently, indicates motion detected
Blue LED	27	Shows video recording in progress

Figure 1 | **Beecam hardware. (a) 3D render of the full setup design**. Beecam is a modular system of 10 behavioural observation units. These each comprise a box attached to a Y-maze, where feeding decisions occur, and are held in place by a big perspex scaffold. Each is associated with a Raspberry Pi with a camera module attached. Breadboards and electrical components not pictured. **(b) Layout of a feeding cage**. Bees in this assay are held individually in custom-made perspex boxes with a transparent Y-maze attached that protrudes into a feeding area. Each arm of the Y-maze is associated with one feeder made from an Eppendorf tube. A Raspberry Pi camera is situated above this region which films feeding events on either choice solution. **(c) Schematic diagram of Raspberry Pi with breadboard and electronic components attached**. Diagram (left) and table (right) illustrate their positioning and function. RPi and breadboard diagram produced with Fritzing software.

using `GaussianBlur()`, and then the amount of change quantified in either feeding region using `absdiff()`. These detection regions encompassed the hole in the relevant feeding tube and the area in and around it, where movement associated with proboscis insertion and drinking was found to occur. All parameters used for motion sensitivity were calibrated through experimental trial and error to optimise the balance between sensitivity and accuracy.

When motion at either feeding region exceeded a threshold, a detection counter was initialised and images from a larger region of interest recorded to a video file that was given a unique name according to the date, time, RPi, and Y-maze arm. This 'filming region' encompassed an area in which the bee's proboscis was visible, typically in addition to several other body parts on the head. The detection counter increased with motion up to a point and declined again after a period of activity. This allowed for the inclusion of a buffer period of up to 6 seconds before filming ends – in practice, we found that feeding bees would be unlikely to evade detection by the algorithm for longer than this length of time. An additional 3s of latency was built into the program signalled by activation of the blue LED. The lights in the circuit provided various other forms of user feedback as to the recording status which are summarised in Table 1.

At the end of the experiment, the button was pressed to release the output stream and clear the GPIOs. Videos could then be manually extracted from the RPi either directly from the SD card or using a USB drive and a VNC viewer.

Pose estimation

To track the interactions of bees with the food, we used DeepLabCut (version 2.3.9) (Mathis et al. 2018, Nath et al. 2019). The model was trained using a total of 849 frames extracted from 190 videos, using 3-5 frames from each. This training dataset included videos taken from a range of animals, lighting conditions, and body positions. For each, we labelled (where visible) the tips of the left and right antennae, the tip of each mandible, the glossa, the labrum, and the point at which the proboscis intersected with the feeding tube (see Table 2).

To train the model, we used a ResNet-50-based neural network for 186000 training iterations. The train and test errors were 3.17 and 12.7 pixels, respectively, and the recording region for both training video and novel footage measured 437 x 437 pixels. The performance of the model was also verified visually by annotating 20 test videos.

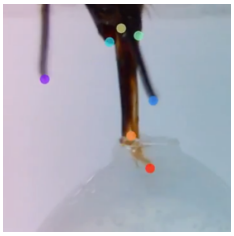
The model was uploaded to Google Drive, as was new experimental video footage. These were accessed together in Google Colab using a script which iterates through unprocessed video in order to analyse it. Output from individual videos was then labelled with metadata and concatenated into a main dataframe using another script.

Behavioural labelling and segmentation

Frames of interest were those in which one or both of the tip of the proboscis and a point of insertion at the feeder were visible with a likelihood of ≥ 0.8. These were assigned a timestamp and a behavioural label (Table 2).

To analyse feeding behaviour, we wanted to perform behavioural segmentation to identify (i) periods of uninterrupted feeding, and (ii) their constituent sips. First, a dataframe was produced by subsetting frames in which the proboscis was present (Table 2, behaviours 2-4), accessible on GitHub as a .csv file. The timestamps of consecutive entries in this dataset were compared for each bee, and individual activity bouts were identified as episodes of proboscis activity that were

Table 2 | Behavioural labelling of frames

Behaviour	Description	Example
No proboscis	Neither point on proboscis detected	
Proboscis out	Proboscis extended but bee is not drinking	
Drinking	Bee is drinking	
Tip obscured	Proboscis probably obscured behind feeder, but bee could be drinking	

separated from one another by 3 or more seconds. Occasional erroneous single frame detections were filtered. The rate of false negatives for the glossa was low, and when an intersection of the tube and the proboscis was detected but not the glossa, it was most often because the proboscis was partially obscured by the feeder (Table 2, bottom row).

Within activity bouts, drinking bursts were then identified by subsetting "Drinking" frames and grouping together those that were temporally contiguous within a maximum of 1 s (i.e. 5 frames). Across consecutive frames in the drinking bursts, the vertical velocity of the proboscis tip was given by $\sqrt{(y_f - y_{f-1})^2}$, where y represents its y-coordinate in a given frame of video, f.

We then sought to determine the distance in pixels associated with rhythmic vertical motion of the glossa so that a threshold could be imposed for sip detection. To do this, we visually inspected the relationship between the extent and direction of vertical motion of the proboscis tip over time $(y_t - y_{t-1})$ to look for clustered bands of data, as well as the counts of vertical displacement of the proboscis tip between frames (Fig. S2). We introduced a sip detection threshold of 40 pixels, meaning that a sip was considered to have been completed when, while the point of intersection with the tube was still present and the glossa had been retracted at least 40 pixels between one frame to the next.

PROTOCOL

In this chapter, I report findings from a proof of concept pilot study ('Experiment 3' in this thesis) in which the behavioural responses of bumblebees to 1M sucrose and 1M sucrose + 10µM quinine, a bitter tastant that is known to be moderately aversive at this concentration (Tiedeken et al. 2014), were monitored over 6 h.

Capture and restraint of bees

Colonies of bumblebees (*Bombus terrestris audax*) were obtained from Biobest (supplied by Agralan, Ashton Keynes, UK). Multiple colonies were used per treatment, and bees were recruited from them in a way that was balanced across treatments and over time. Colonies were maintained in their boxes at room temperature and humidity, fed with BIOGLUC®(Biobest) and supplemented with pollen *ad libitum* (Biobest).

At each round of data collection, ten motivated worker bees were collected in glass vials when they exited the colony. These were then cold anaesthetised until movement had slowed and transferred into individual cages. Recordings were made from a total of 33 individual bees.

Pre-test phase: solutions and feeding tubes

All solutions comprised 1M sucrose (Sigma-Aldrich) made in RO water. Those laced with quinine (Sigma-Aldrich) were produced using an aliquot of the required volume of a 10 mM aqueous stock solution. Experimental solutions were stored in falcon tubes which were frozen at -20°C and defrosted when required. Test solutions were stored in the fridge for up to 3 days before being replaced.

On the day before the experiment, feeding tubes were filled with the test solutions. The side at which each choice solution was located in each choice arena was counterbalanced across the course of the experiment. Once bees were in the cages, the start weights of the feeding tubes were taken. This gave bees a period of around 10 minutes to acclimatise to their environment. Once weighed, these were then inserted at the relevant position in the apparatus.

Initialising and ending data capture

When the tubes and bees were in place, the apparatus was placed into an incubator at 28°C, 60% RH with LED lights installed. The RPis were then plugged in. Once initialised, the red LED light switched on and the data capture phase of the program could then begin by pressing the button. This prompted the green LED to flash several times and then the white LED was illuminated, indicating that initialisation of the data capture phase was complete. Once this had been done for all behavioural recording chambers, the bees were then left for the duration of the experimental choice period.

To terminate the behavioural recording period, the buttons on all units were pressed again. This switched off all LEDs and released any active output streams. The power cables could then be unplugged and the apparatus removed from the incubator. Feeding tubes were then immediately removed and placed on a tray, retaining their positional information, and reweighed. To euthanise the bees, the boxes were removed and placed in the freezer.

DATA VISUALISATION AND ANALYSIS

The individual bee was the unit of analysis throughout, with repeated measures accounted for by including bee ID as a random effect where appropriate.

Cumulative feeding

To generate the per-individual cumulative feeding plots, we first calculated the mean and standard error in cumulative number of bouts, time

feeding, and sips on either solution per individual across 1 h time bins. We then performed 1000 sample Monte Carlo bootstrap resampling using these data and graphed the mean and 95% confidence intervals obtained from the bootstrap samples.

The boxplots accompanying these data show the observed total counts of each behavioural metric per individual on each solution at the end of the experiment. The differences in these relative to the quinine-laced solution (Q – S) were calculated for each individual, assessed for normality using histograms and a `shapiro.test()`, and then used to perform a paired t-test against 0: `t.test(data$diff, mu = 0)`.

Preference indices

Preference indices for the quinine-laced solution were calculated for each bee using the change in volume of each of the choice tubes according to the following formula:

$$\text{PI} = \frac{\Delta \text{ volume }_{\text{Q}} - \Delta \text{ volume }_{\text{S}}}{\Delta \text{ volume }_{\text{Q}} + \Delta \text{ volume }_{\text{S}}}$$

In the same way, the total feeding duration data and sip counts were used to generate preference indices for each individual at the end of the experiment as follows:

$$\text{PI}_b = \frac{\text{Total }_{b,Q} - \text{Total }_{b,S}}{\text{Total }_{b,Q} + \text{Total }_{b,S}}$$

where 'Total' denotes the cumulative count of a given feeding behaviour, b, at the end of the experiment, by solution (Q or S).

Each bee's volume-based preference index was compared to either of the video-derived ones using two Spearman rank correlations: `cor.test(volumePI, durationPI, method = "spearman")` and `cor.test(volumePI, sipsPI, method = "spearman")`. We assessed the strength of each association using the ρ value.

To test whether quinine aversion was significant according to either of the three metrics, the three sets of PI values were each compared against 0 using a series of Bonferroni-corrected Wilcoxon signed rank tests.

The cumulative population-level PI for quinine could be calculated and visualised from the time series video data according to a given behavioural metric (b) with respect to time (t) as follows:

$$\text{PI}_b(t) = \frac{\text{Cumul. }_{Q,b}(t) - \text{Cumul. }_{S,b}(t)}{\text{Cumul. }_{Q,b}(t) + \text{Cumul. }_{S,b}(t)}$$

where $\text{Cumul.}_{Q,b}(t)$ is the cumulative amount of behaviour b occurring at the quinine feeder, $_Q$, up to and including time t across the whole population studied.

Other behaviours

To evaluate bees' sensorimotor responses to quinine vs sucrose, we also looked at the duration of the first bout, the mean bout duration, the mean number of sips per bout, and the average rate of vertical motion of the glossa while drinking on either solution. For each bee, a value of each was calculated per solution. Six bees did not make any detected feeds on quinine, but their sucrose measurements for each metric were included in the visualisation and analysis.

We fit separate mixed models that tested the main effect of solution on each behaviour while accounting for the two such measures taken from each individual by including bee identity as a random intercept. Model fit was assessed visually with the help of the `DHARMa` package diagnostic plots and tests (Hartig 2024).

First bout duration measurements were highly right-skewed. Log transformation did not resolve the issue, and gamma glmms failed the heteroscedasticity test. We therefore fit heteroscedastic `glmmTMB` generalised linear mixed models (Brooks et al. 2017, McGillycuddy et al. 2025) with a gamma distibution and log link function which allowed the dispersion parameter to vary by solution: `glmmTMB(duration ~ Solution + 1|bee_ID), family = Gamma(link = "log"), dispformula = ~ Solution)`. Sips per bout were modelled using a tweedie distribution: `glmmTMB(sips_per_bout ~ Solution + (1|bee_ID), family tweedie(link = "log")`.

Average bout duration was log-transformed and modelled using a linear mixed model using the `lmer()` function from the `lmerTest` package (Kuznetsova et al. 2017), i.e. `lmer(log(av_duration) ~ Solution + (1|bee_ID))`.

The measurements of the mean vertical proboscis motion by solution were approximately normally distributed. We therefore fitted a linear mixed-effects model to these data on the original scale: `lmer(sip_rate ~ Solution + (1|bee_ID))`.

For all models, estimated marginal means and pairwise contrasts were obtained using the `emmeans` package (Lenth 2025). Results are reported on the response scale for ease of interpretation, following back-transformation where relevant using the `type = "response"` argument in `emmeans()`.

Individual preference trajectories

To track the direction and strength of decisions made by individual bees, we plotted two types of preference trajectory over a series of time bins (t), 1h in width.

First, we calculated and visualised the net extent of preferential feeding on the focal quinine solution ('preference vector', PV) as follows:

$$\text{PV}_{duration}(t) = \text{Cumul. }_{Q}(t) - \text{Cumul. }_{S}(t)$$

We also looked at the cumulative net amount of quinine feeding as a proportion of an individual's total feeding at the end of the experiment:

$$\text{PI}_{duration}(t) = \frac{\text{Cumul. }_{Q}(t) - \text{Cumul. }_{S}(t)}{\text{End total }_{Q} + \text{End total }_{S}}$$

Graphs were made using `ggplot2` and exported as svgs using `ggsave`. The plot panels were produced in Inkscape.

REPRESENTATIVE RESULTS

Cumulative feeding

Individual bumblebees could clearly differentiate the two solutions in the two-choice assay (Fig. 2). Differences in cumulative time spent with the proboscis in each feeder (Fig. 2a) and number of sips made (Fig. 2b) set in at the outset of the recording period, and quinine feeding plateaued around halfway through. By the end of the experiment at 6 h, bees had, on average, fed for over 3 times as long on sucrose compared to quinine (mean 165 vs 50 seconds; Fig. 2a; Table 3). They had also sipped more slowly when feeding on quinine: the average bee made nearly 5 times as many sips on sucrose vs quinine by the end of the experiment (mean 198 vs 43.4; Fig. 2b; Table 3). Bees made feeding visits to the sucrose feeder at a constant rate of approximately 4/hour, and had come to visit the quinine-laced solution less frequently by the end of the testing period (mean 24.5 vs 13.1 bouts; Fig. 2c; Table 3).

Table 3 | Paired t-tests of quinine's effect on cumulative feeding measures

Measure	$\bar{x}$ [95% CI]	t_{32}	p	p (adj)
Duration	-116 [-160, -73.2]	-5.49	< 0.001***	< 0.001***
Sips	-152 [-207, -97.8]	-5.70	< 0.001***	< 0.001***
Bouts	-11.5 [-19.2, -3.75]	-3.03	0.005**	0.015*

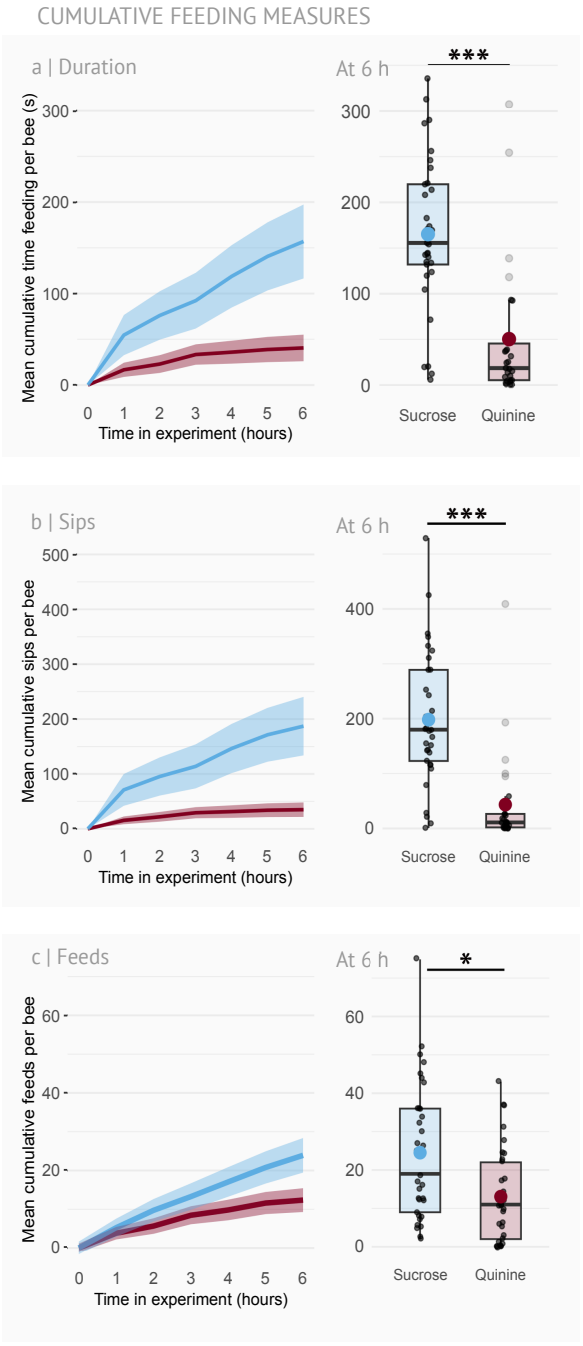


Figure 2 | **Cumulative feeding in experiment 3, a binary choice assay for 1 M sucrose (blue) or 1 M sucrose laced with 10 µM quinine (maroon), by** (**a**) duration, (**b**) sips, and (**c**) number of feeding visits. **Left column**: The average bee consumed less of the quinine-laced sucrose throughout than the pure sucrose, and quinine feeding tended to plateau midway through the experiment. Ribbons show 95% confidence intervals. **Right**: jittered points show the observed cumulative values of each behaviour per bee at the end of the experiment. Their distributions are summarised with boxplots (bars represent the median and IQRs; outliers shown in grey; coloured dots indicate the mean). n = 33. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ after Bonferroni correction for multiple t-tests.

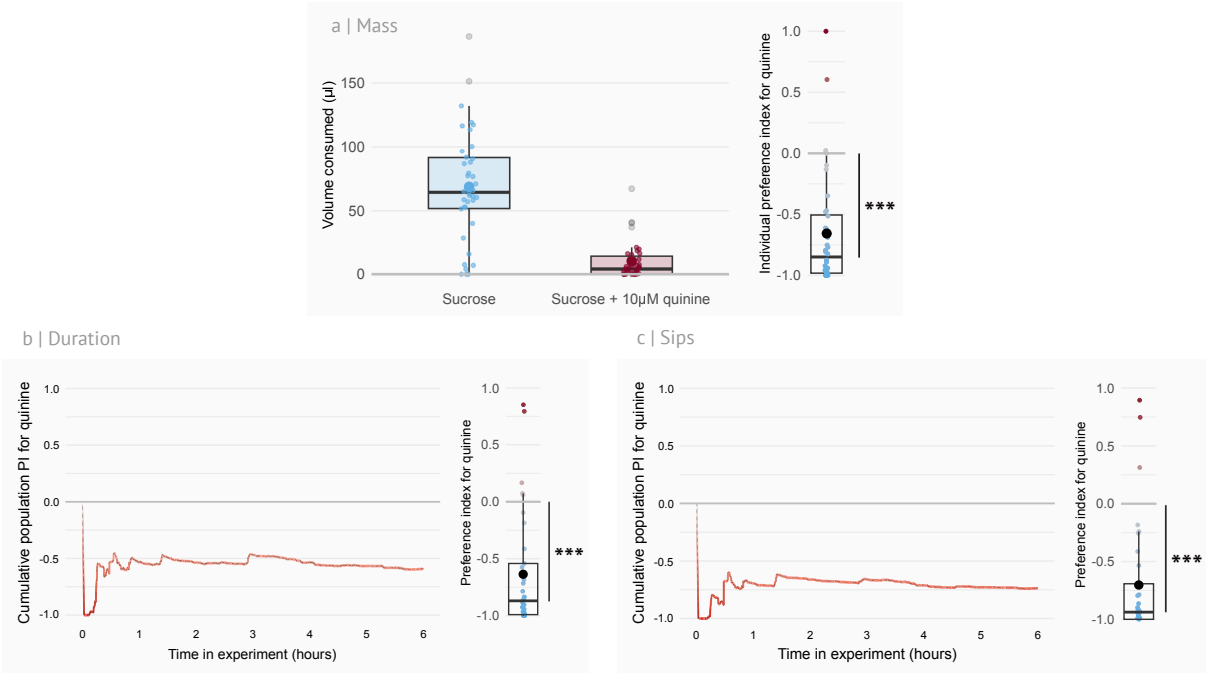


Figure 3 | **Preference indices derived at the end of experiment 3 using choice solution volume change data (top) vs continuously throughout the monitoring period using information from the video data (bottom). Left plots in each facet**: quinine avoidance was expressed at a population level from the beginning of the experiment, as measured either by (a) the amount of each solution consumed at the end of the experiment by each bee, or continuously by (b) time spent feeding on either solution or (c) by the number of sips completed on either food source. **Transparent boxplots, right**: the accompanying boxplots summarise the distribution of the preference indices of individual bees calculated using each of these measures at 6 h (bars represent the median and IQRs; outliers shown in grey; large dots indicate the mean). All preference metrics revealed quinine avoidance, the extent of which was greatest when measured by sips. n = 33 for all. Positive values denote quinine preference, and negative values a preference for sucrose. *** = $p < 0.001$ after bonferroni correction for multiple testing.

Preference index and its evolution

Preference indices of individual bees derived at the end of the experiment from the feeder volume change data (Fig. 3a, left) indicate strong quinine avoidance (Fig. 3a, right; $p < 0.001$; Table 4). The continuous population-level preference index trajectories reveal that this aversive feeding ratio was established early in the experiment (Fig. 3b-c, left facets). Individual preference indices by cumulative time feeding (Fig. 3b, boxplot) and cumulative sips (Fig. 3c, boxplot) at 6 h show a similar degree of quinine aversion to the volume change data. Preference index was lowest when estimated by the number of sips on either solution ($PI_{sips} = -0.938$; Table 4; Fig. 3c).

Table 4 | Wilcoxon tests vs 0 of PI significance

Behaviour	Median PI	Wilcox p	p (adj)
Volume	-0.849	< 0.001 ***	< 0.001 ***
Duration	-0.870	< 0.001 ***	< 0.001 ***
Sips	-0.938	< 0.001 ***	< 0.001 ***

We then tested how well a subject's preference indices calculated from the video-derived duration and sip data tended to correspond to that obtained from the volume change of its feeders. Each PI type was similarly positively correlated with the amount fed (Table 5; transparent boxplots, Fig. 3), but duration-based PI was perhaps a slightly closer fit that the amount of detected sips.

Table 5 | Spearman's rank correlations of PIs derived from sips and duration vs amount of food consumed

PIs compared	Spearman's ρ	p
Duration vs volume	0.549	< 0.001 ***
Sips vs volume	0.533	0.0014 **

Other related behaviours

To see how sensitive our assay was to detecting aversive taste, we had a closer look at some structural features of feeding behaviour.

The duration of a bee's first bout of feeding activity was strongly reduced when quinine was present: initial bouts lasted 18 times longer on average when bees were feeding on sucrose (25.7 vs 1.42 seconds, 95% CI ratio: 6.13–53.1, p < 0.001; Fig. 4a; Tables 6 and 7). Quinine feeding bouts were also significantly shorter in duration on average throughout by about 5-fold (Fig. 4b; 7.24 vs 1.39 seconds, 5.2×, p < 0.001, Tables 6 and 7) and

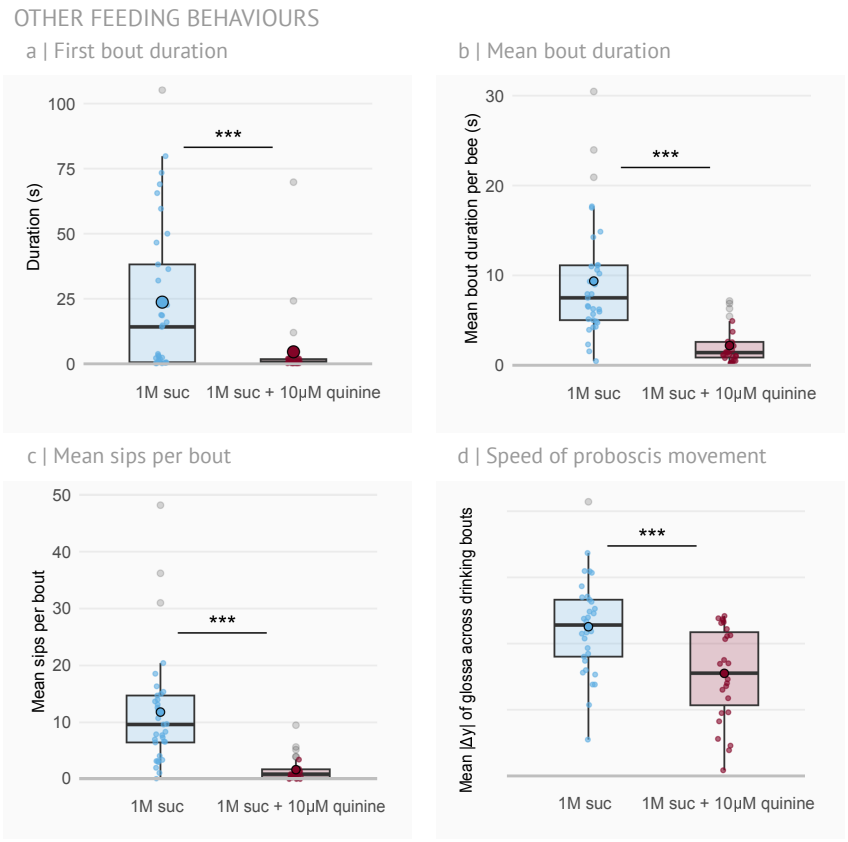


Figure 4 | **Other taste-related changes to feeding behaviour in experiment 3**. Each graph compares the behavioural response to sucrose (left, blue) vs quinine-laced sucrose (right, maroon). (**a**) The first drinking bout from either solution was significantly shorter when quinine was present in the solution (though with three notable outliers), which (**b**) was also the case during subsequent quinine feeds. (**c**) Bees also made fewer sips per bout from the quinine-laced solution. (**d**) The proboscis tip moved more slowly on average during completed sips from quinine-laced food. Data points represent the value of the feeding metric per solution per individual. Outliers are shown in grey. Bars in the boxplots represent the median and IQRs, and coloured dots indicate the mean. n = 33 for all; *** = $p < 0.001$.

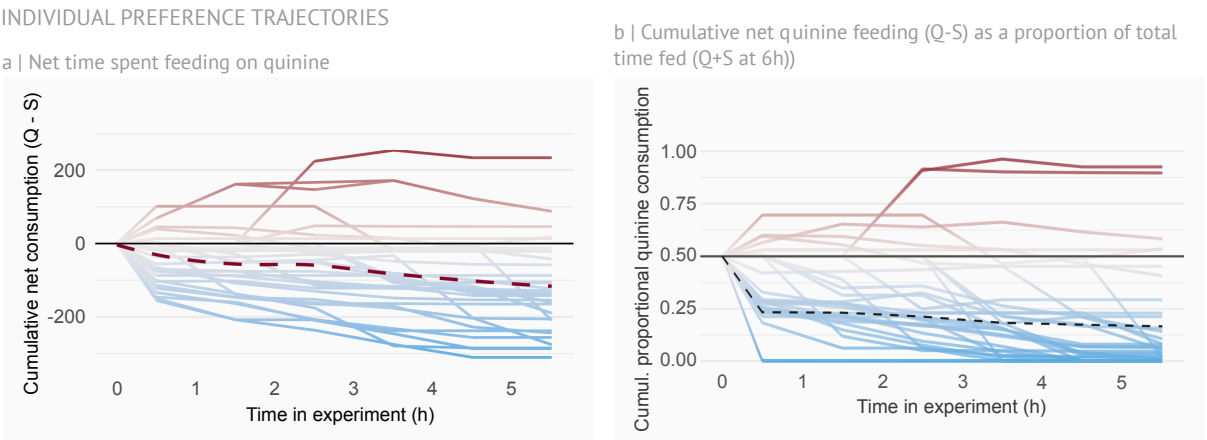


Figure 5 | **Preference trajectories from individual bees within experiment 3**, calculated across a series of 1h-wide time bins using the feeding duration data. Solid lines show (**a**) the cumulative net amount of feeding on quinine in seconds, and (**b**) the same, expressed as a proportion of all the subject's feeding by the end of the experiment. The hue and intensity of the trajectories denotes their direction and strength, respectively, where blue indicates a preference for the pure sucrose and red for quinine. Dotted lines show the mean response at the population level across the time bins. Most bees came to forage near-exclusively on the food without quinine, but there were some notable exceptions. n = 33

consisted of about 9 times fewer sips (Fig. 4c; 10.4 vs 1.17, 8.82×, $p < 0.001$, Tables 6 and 7). Of the completed sips individuals made on either solution, they were slower on average when sips were made from the quinine-laced food (Fig. 4d; 31.0 vs 45.1 pixels/second, $p < 0.001$, Tables 6 and 7).

Table 6 | Group emmeans showing average amount of behaviour by solution, with confidence interval

Behaviour	Solution	Mean	95% CI
First bout (s)	S	25.7	11.9–55.6
First bout (s)	Q	1.42	0.67–3.03
Average bout (s)	S	7.24	5.27–9.96
Average bout (s)	Q	1.39	0.98–1.98
Sips per bout	S	10.4	7.61–14.1
Sips per bout	Q	1.17	0.74–1.86
Sip speed (pixels)	S	45.1	40.2–50.0
Sip speed (pixels)	Q	31.0	25.6–36.5

Table 7 | Contrast ratios/difference and significance

Contrast (S vs Q)	Est.	95% CI	p
First bout	18 ×	6.13–53.1	< 0.001***
Average bout	5.21 ×	3.31–8.18	< 0.001***
Sips per bout	8.82 ×	5.57–14.0	< 0.001***
Sip speed	+14	6.98–21.1	< 0.001***

Individual preference trajectories

On average, bees steadily decreased their net consumption of quinine over time (Fig. 5a) according to a pattern of preferential sucrose feeding that was established early in the experiment (Fig. 5b), but with a small number of notable outliers.

DISCUSSION

We developed a novel technique to record and analyse the feeding behaviour of bees over time in a two-way choice setting. We then verified the method by demonstrating that 10 μM quinine in 1M sucrose is distasteful to bumblebees by a variety of behavioural metrics. From the beginning bees could clearly differentiate between the solutions and at a population level they maintained a strong level of aversion throughout the monitoring period. Near-total quinine avoidance was the modal response although there was a small amount of behavioural variation. By the end of the recording period, bees had consumed a significantly lower volume of quinine-laced food, spent significantly less time feeding on it, and made proportionately fewer sips from it too. Our data show that this assay is sensitive to the detection of aversive feeding in bees and can reveal information about the dynamics of feeding decisions over time.

Most bumblebees found 10μM quinine in 1M sucrose more distasteful than predicted

Quinine has been shown in several studies to be distasteful to bees and to facilitate aversive learning at a range of concentrations (Chittka et al. 2003, Mustard et al. 2012, Wright et al. 2010, Ma et al. 2016). Its detection threshold in 0.5M sucrose by *Bombus terrestris* was previously determined using a caged assay to be somewhere between 1-10 μM (Tiedeken et al. 2014). At 10 μM, the bees in that study consumed about half as much of the quinine-laced solution as the unadulterated 0.5M sucrose to give a PI of approximately -0.4.

We expected, if anything, to find comparatively blunted quinine detection ability given our use of doubly concentrated sucrose (1 M) with its increased potential for sugar masking (von Frisch 1967, Gegear et al. 2007, Köhler et al. 2012, Cocco & Glendinning 2012). Instead, we observed bees consuming about 5x less of the quinine-laced food and our calculated preference indices conveyed a greater degree of quinine aversion. Whatever the cause of this discrepancy, it seems to highlight the sensitivity of this setup and its detection power. Previous studies found that bees reject unpalatable food less often when harnessed (Ayestaran et al. 2010) in comparison to those whose movement is less artificially constrained (Ma et al. 2016, Chittka et al. 2003, Avarguès-Weber et al. 2010, de Brito Sanchez 2011, Rodríguez-Gironés et al. 2013, de Brito Sanchez et al. 2015). This is encouraging from a methodological perspective as it suggests that this assay is configured in a way that promotes and captures feeding decisions that are likely to be more field-relevant.

There were two notable outlying bees that sampled from both feeders but chose mainly to feed on the quinine-laced solution, and they each completed an uninterrupted quinine feeding bout of over one minute in length that was reflected in the sip and volume data. We verified this by inspecting the video recordings. The concentration of quinine used (10 μM) is orders of magnitude below those typically used as a negative control in other feeding experiments, and within one order of magnitude of the reported detection threshold in dilute 0.5 M sucrose (Tiedeken et al. 2014) so perhaps this was a product of individual variation in bitter taste sensitivity, potentially coupled with differences in learning ability (Sezen et al. 2021, Ferguson et al. 2001).

Bees seemed to show some latency in avoiding to visit the quinine-laced food

The number of feeds at either food source will inevitably be a lower resolution metric of differential reward valuation because every individual

instance of proboscis contact with the solution is weighted equally regardless of the size of the feeding event. Nonetheless, we expected visitation dynamics to somewhat resemble those obtained during differential go/no go conditioning assays.

We observed that bees fed for significantly less time on quinine from the moment they first encountered it, and the sips they made were proportionally fewer in number and slower, too. However, on average they only began to visit this feeder significantly less about 2-3 hours into the test phase. Ostensibly, they may therefore have shown some latency in their formation of an aversive associative memory with the quinine-laced solution in this context.

Bees have the capacity to learn and remember spatial relations between food sources (Brown & Demas 1994, Ushitani et al. 2016, Patel et al. 2022), and Y-mazes have been used for differential conditioning in bees in conjunction with odours and colour cues (Carcaud et al. 2009, Nouvian & Galizia 2019). Bees have also been successfully trained to expect a sucrose reward from one arm of a Y-maze in the absence of any supplementary sensory information (Giurfa et al. 2001) so we expected they would be able to learn to visit the sucrose arm despite the paucity of accompanying floral cues and salient visual information in the experimental environment.

Our bees appear from these data to have been relatively slow to do so. However, in contrast to binary go/no go experiments, our bees were not forced to make active choices but were given *ad libitum* access to each unconditioned stimulus, and the punishment analogue was a lot less concentrated and delivered in 1 M sucrose. Long, decisive, uninterrupted bouts of feeding will also have been processed as single events, whereas individual instances of flickering proboscis contact with the deterrent solution will have been counted separately. Additionally, the average responses to the solutions do not reveal the different strategies of particular bees towards feeding on the quinine-laced food: for example, in addition to the two quinine-favouring outliers, five bees did not bias their feeding towards the sucrose feeder early on but instead fed relatively indiscriminately at the beginning of experiment. They gradually favoured it over time, perhaps having experienced milder aversion to it.

In sum, the cumulative feeding visit data probably can't therefore be used to measure the dynamics of aversive learning with accuracy but nonetheless confirm that 10 μM quinine is aversive to bees when added to the sucrose solution.

Of the video-derived data, feeding duration produced the most accurate preference measurements

The per-individual preference index values calculated at the end of the experiment using the feeding duration data and the sip frequency data both correlated well with those derived from the volume change measurements of the respective feeders. These two measures obviously covary as they are derived from video recordings of the same feeding events, but nonetheless suggest that the sip detection algorithm worked reasonably well. Quantitatively, feeding duration corresponded slightly more closely to the volume change measurements and produced a median PI value that was between that of the volume and sip data in magnitude. We therefore decided to use this form of time series data to create the individual preference trajectories in Fig. 5, and also streamlined our assessments of individual and population-level preference over time in the subsequent chapter by focussing on feeding duration.

Assay limitations and how to mitigate them

Of the strongly quinine-averse bees, six did not make any detected quinine feeds whereas all test subjects were recorded consuming sucrose. Recent work shows that bumblebees can detect quinine using their antennae (Harrap et al. 2025) so bees were probably antennating the solution and avoiding it. The antennal tips are tracked by our trained model but the data extraction pipeline had not been set up to analyse these movements. Were the setup to be used in future specifically for studying taste responses to bitter compounds, it would perhaps be useful to adapt the scripts to this end.

The pose estimation model was trained using footage that was collected when the lights in the incubator were on. Were we to incubate bees according to a light/dark cycle, we would not currently be able to capture data in the dark. For our purposes here this does not matter, but if this method was applied to study behaviour over longer timescales the pose estimation algorithm would need to be retrained.

Unfortunately bees often tried to escape through holes at the end of the Y-mazes which resulted in the capture of a lot of irrelevant video material. It was filtered out easily through our pose estimation and behavioural detection algorithms, but the process was quite a bit more computationally intensive than would otherwise have been necessary. Our pipeline relied on Google Colab, but ideally users would have access to high performance computing resources.

The setup therefore has its limitations, but we are nonetheless hopeful that it will provide us

with a pivotal tool for tackling questions about the subtle and temporally-dependent effects of pharmacological agents in nectar.

Potential future uses and suggestions

This technique allows for the automatic capture of time series data which is rich in information about individual behavioural responses to a two-way feeding choice across extended timescales. This could provide researchers with the possibility to answer a variety of research questions relating to bee nutritional homeostasis and nutrient regulation using a method analogous to existing setups but which yields greater temporal and behavioural resolution while necessitating many fewer test subjects than some methods. We foresee that the application of this technique to the following behavioural phenotypes could be particularly instructive.

Post-ingestive learning: bees can learn which food sources to seek and avoid through both their tastant qualities and through the post-ingestive consequences of consuming them. This assay could provide a set of tools for distinguishing these processes from one another, such as by comparing the taste-oriented metrics (e.g. first bout duration, number of sips in first bout) to the patterns of visitation to the feeder over time. In a similar way, and for similar reasons, it could be used to investigate the acquisition of addictive feeding preferences.

Drug self-administration: the motivation to self-administer an addictive drug will fluctuate over time according to an individual's homeostatic state, which itself will depend on patterns of previous drug self-administration. Bouts of behavioural evidence of this sort are therefore likely to be transient, subtle, and difficult to interpret meaningfully, especially at a population level, in the absence of contextual information about their temporal patterns.

Understanding phenotypic variation and identifying polymorphisms: bees may conceivably differ in meaningful ways in their mechanisms and expression of feeding responses. However, of the existing methods that analyse feeding responses at the individual level, they tend not to yield sufficiently detailed information to be able to quantify or characterise the variation. Our assay may provide the means to discern subtle but meaningful signals among what appears as noise.

Conclusions

This protocol can be used to measure a variety of bumblebee feeding responses to a set of two choice solutions continuously across unbounded timescales. It is automatic, requires minimal test subjects, and supplies high resolution, information-rich datasets that can be used to track responses to solutions of interest at the individual and population levels. We demonstrate that the technique can detect aversive taste at population and individual levels with sensitivity, and suggest fruitful applications in a range of research areas.

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Data availability

All scripts and materials to reproduce the setup are available at https://github.com/annadorling. The RPi software repo contains the code for RPis to detect motion in and around the entrance of the feeders and record feeding events (cameracontrols.py). The systemd file, Beecam.service, can be used to make this program run on boot. Laser cutting .dxf templates for reproducing the hardware can be found in a .zip file in the Beecam-hardware repo, with instructions on the materials required. Two publicly available Google Colab notebooks were used to process the videos in Google Drive: one to label bodyparts using DLC, and another to concatenate the output .csv files into a main dataframe. The output dataframe generated from experiment 3, along with the blinds .csv file and the .Rmd script that was used to extract and visualise the behavioural information from them are available at Quinine-beecam.

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SUPPLEMENTARY INFORMATION

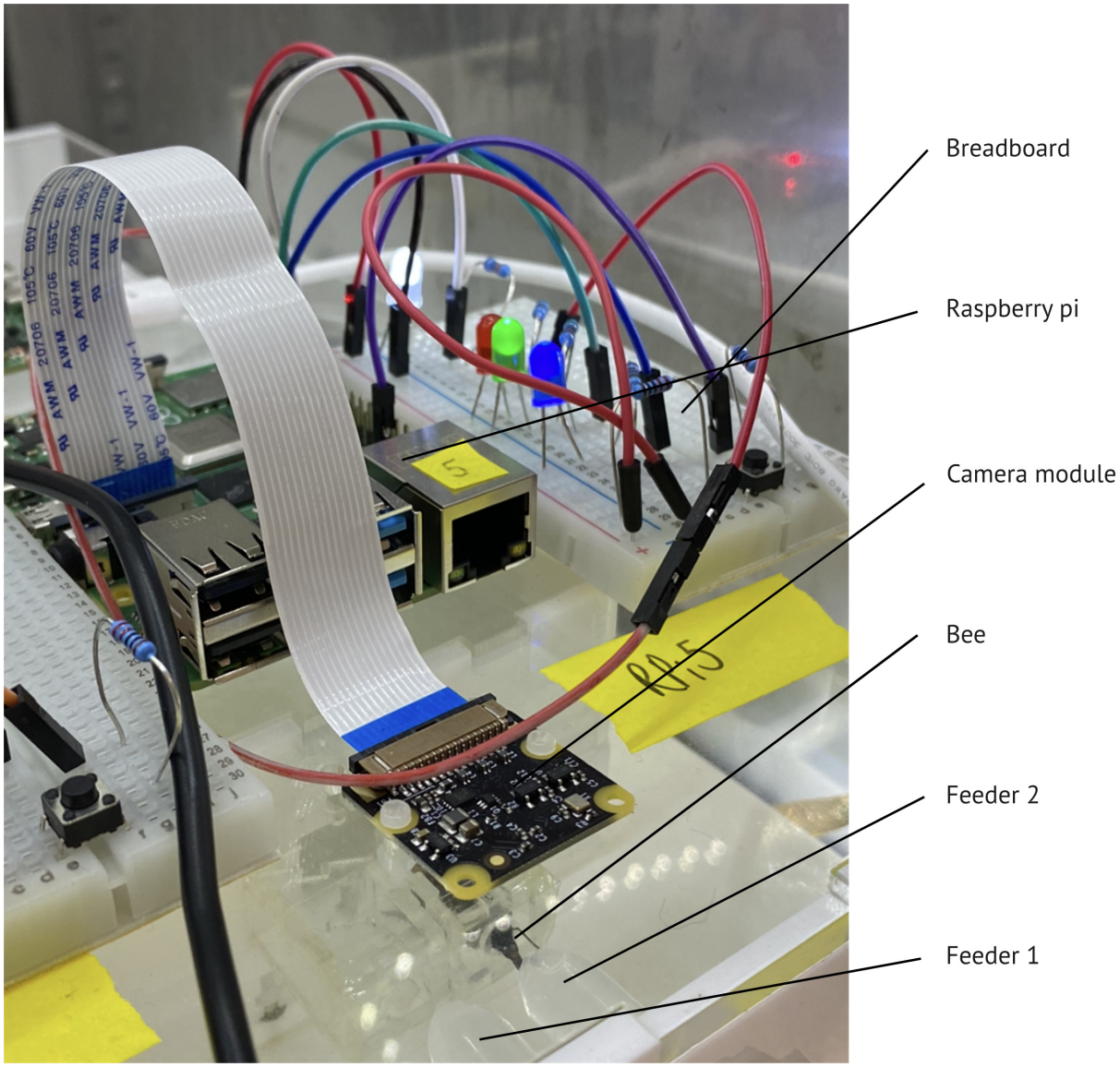


Figure S1 | **Labelled photo showing components of the apparatus**. Bees are presented a choice betwen two food sources delivered in eppendorf tubes at the end of either arm of a camera-monitored y-maze.

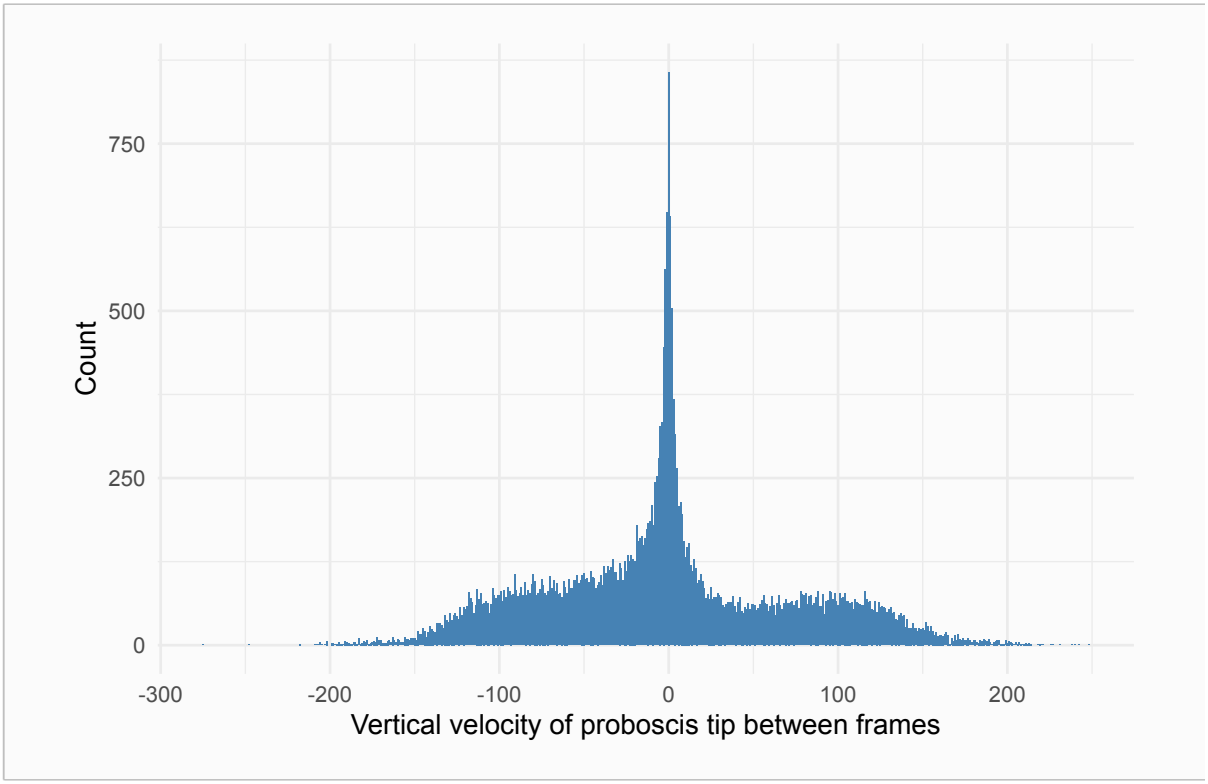


Figure S2 | **Distribution of vertical velocity values between frames during drinking bursts**. The modal response of the proboscis tip between frames of video was a lack of movement. Either side of this peak are sip signals centred at around -80 and 100 pixels

Stats tables

Table S3a | First bout duration model

```
fbd_mod <- glmmTMB(duration_sec∼Solution + (1|bee_ID), family = Gamma(link = "log),
dispformula = ∼ Solution)
summary(fbd_mod)
```

Fixed effects	**Estimate (log scale)**	**SE**	**z**	***p***
Intercept	3.25	0.394	8.24	< 0.001***
SolutionQ	-2.89	0.551	-5.25	< 0.001***
Random effects	**Variance**	**SD**		
bee_ID	1.16	1.08		
Dispersion model	**Estimate**	**SE**	***z***	***p***
Intercept	-0.823	0.240	-3.43	< 0.001***
SolutionQ	0.825	0.582	1.42	0.156

Table S3b | Average bout duration model

```
abd_mod <- lmer(log(av_duration)∼Solution + (1|bee_ID))
summary(abd_mod)
```

Fixed effects	**Estimate (log scale)**	**SE**	**df**	**t**	***p***
Intercept	1.98	0.159	56.9	12.5	$< 0.001^{***}$
SolutionQ	-1.65	0.220	30.0	-7.49	$< 0.001^{***}$
Random effects	**Variance**	**SD**			
bee_ID	0.125	0.353			
Residual	0.709	0.842			

Table S3c | Sips per bout model

```
sips_per_bout_mod <- glmmTMB(sips_per_bout ∼ Solution + (1|bee_ID), family =
tweedie(link = "log"))
summary(sips_per_bout_mod)
```

Fixed effects	**Estimate (log scale)**	**SE**	***z***	***p***
Intercept	2.34	0.158	14.8	$< 0.001^{***}$
SolutionQ	-2.18	0.234	-9.30	$< 0.001^{***}$
Random effects	**Variance**	**SD**		
bee_ID	0.285	0.534		

Table S3d | Average speed of glossa motion while drinking model

```
sip_speed_mod <- lmer(sip_speed ∼Solution + (1|bee_ID))
summary(sip_speed_mod)
```

Fixed effects	**Estimate**	**SE**	**df**	**t**	***p***
Intercept	45.1	2.45	57.3	18.4	$< 0.001^{***}$
SolutionQ	-14.0	3.44	31.3	-4.08	$< 0.001^{***}$
Random effects	**Variance**	**SD**			
bee_ID	24.9	4.99			
Residual	174	13.2			

Chapter 4 | Using camera-monitored feeding cages to study addictive behaviour towards nectar drugs

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Abstract

Some plant-produced alkaloids such as nicotine and caffeine have pharmacological targets in animal nervous systems. At high concentrations these compounds can cause paralysis and death but in low concentrations they can alter the brain's state and modify the processes of learning and memory. Such compounds have been found in low concentrations in floral nectar. Several studies have observed that caffeine, nicotine, and compounds like nicotine impact learning and memory in bees. Long-term exposure to these compounds could even produce addiction in pollinators, but this remains untested. In this work we explored whether a selection of nectar-relevant drugs that influence cholinergic signalling in the insect brain can induce addictive behaviour in bees by tracking their preferences for drugs in 1M sucrose vs unadulterated 1M sucrose over 24h using the assay described in Chapter 3. Nicotine-laced nectar was preferentially consumed towards the beginning of the monitoring period. This effect was stronger in bees previously exposed to the drug and subjected to a withdrawal period. Pre-exposure to and withdrawal from arecoline, another natural nAChR agonist, produced similar patterns of responding. Responses of bees to thiamethoxam, a neonicotinoid pesticide, were more ambiguous with respect to this hypothesis, and bees did not express preferences for or addiction to caffeine or scopolamine in this context.

INTRODUCTION

Many flowering plants recruit bees as pollen vectors, often from within competitive floral landscapes, by attracting them with various floral signals and nectar rewards. Nectar is a source of nutrients such as carbohydrates. Pollinators are finely adapted to assessing the quality, quantity, and probability of encountering rewards. They are adept at learning and remembering where to find rewarding nectar. Nectar's chemical composition likely therefore experiences selective pressure to make it easier for bees to learn and remember: plants offering more valuable nectars are more successful at conditioning pollinators to visit conspecific flowers which promotes their outcrossing (Chittka & Schürkens 2001). Interestingly, certain flowering plants express nectar spiked with low concentrations of potentially toxic secondary metabolites such as nicotine and caffeine. These could hypothetically serve various ecological functions within plant-pollinator relationships (reviewed by Stevenson et al. (2017)). Some can interact with receptors in the insect nervous system (Mustard 2020) giving them the potential to manipulate pollinator behaviour.

Various *Coffea*, *Citrus* and *Tilia* species produce caffeinated nectar (Stevenson et al. 2017, Wright et al. 2013, Naef et al. 2004), which recent evidence shows may be preferred by bees (Singaravelan et al. 2005, Couvillon et al. 2015). Caffeine antagonises adenosine receptors. In mammals, adenosine is known to act as an inhibitory neuromodulator (Dunwiddie 1985, Williams 1990). Caffeine is thought to increase the excitability of Kenyon cells (KCs) principally by inhibiting the neuromodulatory action of adenosine in the projection neurons (Wright et al. 2013), helping to depolarise the KCs towards their action potential threshold and thereby rendering insects more sensitive to nicotinic acetylcholine receptor (nAChR) activation in response to sensory stimulation (Perez-Orive et al. 2002). Blocking the effects of ACh on KCs with an nAChR antagonist reverses the effect (Wright et al. 2013). Caffeine thus seems to facilitate the formation and long term retrieval of memory traces such that bees can be over twice as likely to remember a conditioned association at 24 h (Wright et al. 2013). Ultimately this may improve the floral constancy of bees; this benefits plants, but potentially comes at a foraging efficiency cost to bees (Couvillon et al. 2015).

Nicotine is an alkaloid bees may encounter in the nectar of certain *Nicotiana* species (Adler et al. 2006, Kessler et al. 2012, Singaravelan et al. 2005, Tadmor-Melamed et al. 2004, Tiedeken et al. 2014) and which also elicits preferential and perseverative feeding behaviour at nectar concentrations (Singaravelan et al. 2005, Baracchi et al. 2017). It is an agonist of acetylcholine receptors which are found throughout the insect nervous system (Grünewald & Siefert 2019, Giordani et al. 2023). ACh mediates cognition, sensory processing, and motor function in animals, and is the main excitatory neurotransmitter in insects (Gauthier 2010). ACh binds to nicotinic and muscarinic acetylcholine receptors (nAChRs and mAChRs) which are distributed widely in in-

sect neural and non-neuronal tissue. In the insect brain, the KCs receive cholinergic input from projection neurons (Yasuyama & Salvaterra 1999) and communicate with mushroom body output neurons via nAChRs (Barnstedt et al. 2016, Gu & O'Dowd 2006). Thus, it is possible that any drug in food that can influence cholinergic signalling could manipulate reward learning in bees if they have receptors that are sensitive to it. In mammals, highly nicotine-sensitive acetylcholine receptors are also expressed in dopaminergic neurons involved in this process (Miwa et al. 2011, Biasi & Dani 2011, Biasi & Salas 2008). Compounds that are directly able to usurp the dopaminergic mechanisms of reward encoding may have the potential to be addictive.

Most drug addiction research in animal models has involved training rodents to self-administer the compounds intravenously or depriving them of food or water to overcome bitter taste aversions. Bees have poor bitter taste acuity (Parkinson et al. 2023, Guiraud et al. 2018, Tiedeken et al. 2014, Kessler et al. 2015, de Brito Sanchez et al. 2005, 2014) and will naturally encounter neuroactive compounds in food (Mustard 2020), hypothetically making them vulnerable to repeated drug exposure. In humans and animal models, recurrent nicotine use produces persistent, hyper-rewarding memories. When the brain is exposed to nicotine over the long-term, it stimulates a compensatory change in the number, sensitivity and composition of receptors expressed in the brain (Biasi & Dani 2011). These changes can alter the brain's chemical homeostasis. A hallmark addictive behaviour is withdrawal-induced drug-seeking. Under nicotine withdrawal, drug-seeking motivation increases in a bid to restore homeostatic balance. We therefore hypothesise that repeated exposure to nAChR agonists could give nectar addictive properties to bees.

Other nAChR agonists may occur naturally in nectar, and it seems reasonable to predict they could also be addictive to bees. For instance, arecoline is a compound that may be present in the nectar of the palm *Areca catechu* and is the active ingredient in the betel nut, which is chewed addictively by millions of people in South-Eastern Asia. It agonises relevant subpopulations of nAChRs that are expressed in the brain and involved in learning and addiction (Papke et al. 2015, Winger 2021) and can induce addictive patterns of gene expression in mice (Li et al. 2025), potentially with nicotine-like behavioural effects (Winger 2021, Li et al. 2025, Liu et al. 2025).

Pesticides such as neonicotinoids target nAChRs, and when used as systemic pesticides, they are found in the nectar of flowering crops. They bind to nAChRs with very high affinity (Tomizawa 2013, Casida 2018). Bees cannot taste them (Parkinson et al. 2023) but very low yet field-relevant concentrations of thiamethoxam (TMX) and imidacloprid can produce preferential feeding behaviour due to their pharmacological action on the brain (Kessler et al. 2015). As they tend to have lots of adverse side effects (Siviter et al. 2021), addiction to these drugs could be detrimental to bee populations.

Besides caffeine and nAChR agonists, drugs that modulate the cholinergic mechanisms of reward in other ways could perhaps also be addictive. Various *Datura*, *Lycium*, *Brugmansia* and *Hyoscyamus* species produce scopolamine-laced nectar at concentrations from $\sim$10 μM to 1.3 mM (Boros et al. 2010, Kerchner et al. 2015). It antagonises mAChRs. Unlike nAChRs, mAChRs are G-protein coupled receptors, and they are present at much lower densities in the insect CNS (Trimmer 1995). There are excitatory and inhibitory subtypes but both are metabotropic. This form of neurotransmission is slower and modulates synaptic plasticity. Scopolamine-sensitive mAChRs are expressed in the KCs and antennal lobes (Blake et al. 1993, Rozenfeld et al. 2019). They appear to be less critical to reward learning than nAChRs, but may be important for memory retrieval and shaping KC morphology with foraging experience (Silva et al. 2015, Bielopolski et al. 2019, Weinberger 2004, Ismail et al. 2006, Gauthier et al. 1994, Lozano & Gauthier 1998, Feiro & Gould 2005, Hefco et al. 2003, Liao et al. 2019). Detzel & Wink (1993) found that bees avoided scopolamine-laced food, but two studies report that it elicits appetitive feeding in bees (Sculfort et al. 2021) and influences dendritic morphology in the MBs in a way that potentially improves foraging efficiency by "enhancing normal developmental synaptic pruning" (Dobrin et al. 2011).

We designed a series of experiments that used beecam to examine how drug preferences may develop and whether drug pre-exposure may produce addictive patterns of feeding.

In experiment 4a, we explored whether preferences for food laced with 25 μM nicotine would develop in bees that were naive to the drug vs in those that had experienced 3 days of exposure to the solution followed by a 24h withdrawal period. We predicted that bees in both groups would express preferential nicotine feeding on average, but were not sure when to expect such an effect to be detectable: drug preferences could conceivably be fixed and most salient by the end of the experiment, or expressed only transiently if subject to homeostatic mechanisms. We therefore first compared whether nicotine-naive and pre-exposed bees given a choice to feed on food laced with the drug over 24 h had consumed significantly more

of it than the undrugged food option at the end of the experiment. We then looked at whether nicotine intake was biased towards the beginning of the monitoring period by testing for a reduction in time to 50% consumption of the nicotine-laced solution compared to sucrose, and for higher rates of feeding on this solution across a set of time bins. First bout duration can also be a useful metric of preference, so we examined whether initial feeding bouts on nicotine-laced food were significantly longer in duration on average than for pure sucrose.

In accordance with the addictive hypothesis we expected to see clearer initial preferences for nicotine under withdrawal, and thus assessed whether the previously described effects were stronger in the pre-exposed group compared to the drug-naive bees. Additionally, we probed for inter-individual variability in responses to nicotine. To do so we did not perform null hypothesis testing but visually inspected the distribution of individual preference trajectories over time. We were curious to see whether the bees' responses to nicotine would bifurcate somewhat, particularly after pre-exposure, potentially as a consequence of variation in susceptibility to the effects of addictive drugs.

In a subsequent experiment (experiment 4b), we pre-exposed bees for three days to solutions containing either 10 μM arecoline, 35 nM TMX, 100 μM caffeine, or 80 μM scopolamine, and, after 24 h withdrawal, used the same procedure to screen subjects for preferential feeding on the drugged solution. If these other nectar-relevant drugs that influence cholinergic signalling in the insect brain could also induce addictive behaviour in bees, we expected to find patterns of responding that were the same as those obtained from the nicotine pre-exposed bees in experiment 4a.

METHODS

Experiments

We used two experiments to explore whether exposure to putatively addictive nectars can generate feeding behaviours symptomatic of addiction. In experiment 4a, bees were monitored over 24 h for their feeding encounters with 1 M sucrose or an equivalent solution laced with our model drug, nicotine. We looked at an assortment of feeding responses to either solution at the collective and individual levels and assessed if these were influenced by nicotine pre-exposure and withdrawal. In experiment 4b, we employed the same pre-treatment and testing regime to investigate whether pre-exposure to other potentially addictive nectar drugs can influence these behaviours. Table 1 summarises these treatment and testing schedules and provides the sample size of each group. Bees were each assigned to only one of the treatment groups and underwent one behavioural recording session. The individual bee thus constituted the unit of analysis.

Test subjects

Buff-tailed bumblebees (*Bombus terrestris audax*) were obtained and maintained for use in our experiments as described in the previous chapter.

The apparatus has a maximum capacity of 10 bees per recording session. In the present experiments, data capture occurred in 24 h windows. The collection and pre-treatment of bees was staggered to account for this such that 12 foragers were recruited for pre-treatment each day. To do this, worker bees were captured in glass vials as they left the colony.

Experiment 4a was performed in Aug–Sep 2024, and experiment 4b in Nov 2024–Jan 2025. Five and eight distinct colonies were used respectively.

Solutions

Stock drug solutions of arecoline hydrobromide, caffeine, nicotine hydrogen tartrate salt, scopolamine hydrobromide and thiamethoxam (all sourced from Sigma Aldrich) were made in RO water and frozen. To produce the 1M sucrose solutions, reagent-grade sucrose was dissolved in RO water and, where necessary, the required quantity of stock solution was added during the dilution to make the final drug concentrations (Table 1). Concentrations of TMX and caffeine were chosen to reflect those known to produce behavioural effects in bees (Arce et al. 2018, Thomson et al. 2015, Arnold et al. 2021, Tiedeken et al. 2014, Wright et al. 2013). In the absence of this information for the other drugs, we decided to use the reported scopolamine concentration in *Lycium barbarum* nectar (∼79 μM) (Kerchner et al. 2015) and produced a coarse estimate of the arecoline concentration to use by scaling reported values in the mammalian pharmacological literature. We used 25 μM nicotine for its nectar relevance and for the sake of congruity between experiments.

Pre-exposure treatments

After capture, bees were placed on ice until their movement had slowed, and transferred to one of two feeding cages identical to those used in the preference experiment (2a) in chapter 2 (Fig. 1), each containing 6 individuals. Treatment assignment was randomised across the experiment and between the cages.

Once caged, bees were given *ad libitum* access to a pre-weighed quantity of the pre-exposure solution (Table 1, days 1-3). This was delivered in a

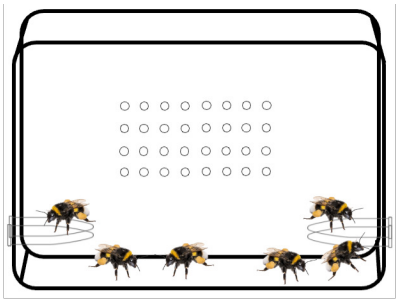


Figure 1 | A feeding cage. Bees were held in ventilated plastic boxes for the duration of the pre-exposure and withdrawal phases, and given access to the test solution in feeders made from modified eppendorf tubes.

set of four 2 ml eppendorf tubes, each with a series of four holes drilled along the side through which the proboscis could be inserted. Subsequent deliveries of a known mass of fresh pre-exposure solution occurred at 24 h and 48 h. At the end of each 24 h feeding window the mass difference of the feeders was determined. At 72 h, drug access was withdrawn: all bees received a final feed of 1 M sucrose for 24 h. They were each then removed from the feeding cages and transferred to an individual observation chamber at 96 h as described in chapter 3.

Data capture

Feeders containing the choice solutions were filled, weighed, and assigned to either y-maze arm via pseudorandomisation as described in chapter 3. Data capture was initiated at a noted time and ended exactly 24 h later. Ten minutes of changeover time was accumulated with each iterative round of data collection, but all recording sessions were started between 10 am and 12 pm. Between uses, boxes were washed with odourless dish soap (Ecover zero) to remove scent markings and dried thoroughly.

Data analysis

Dataframes were extracted from the videos according to the pose estimation, behavioural labelling, and thresholding methods in the previous chapter. The individual bee was the unit of analysis throughout, with repeated measures accounted for by including bee ID as a random effect where appropriate.

Cumulative curves

The mean cumulative curves were generated as per the methods chapter.

For each bee, the amount of time spent feeding on each solution by the end of the experiment and the cumulative number of detected feeding events on each solution were identified. Additionally, 'T50' point timestamps were identified for each bee by solution which marked the start of the bee's feeding bout during which 50% of its cumulative behavioural total for that solution was reached. They were used to investigate initial rate of consumption. The means of these times are visualised as vertical dotted lines on the cumulative curves.

To test for differences in both the extent and rate of feeding by solution, we made linear mixed effect models using the `lme4` package (Bates et al. 2015) with a `(1|bee_ID)` random effect to account for the dual solution measurements per bee. Each model analysed one of the four behavioural metrics (total duration of feeding at 24h, total feeding events at 24h, $T50_{duration}$, or $T50_{bouts}$). In experiment 4a the choice treatments were analysed separately to the no choice group to avoid issues with rank deficiency associated with the

Table 1 | Treatment schedules. The solutions on which bees were fed at each stage in the experiment, and the final number of recordings made per group.

Experiment 4a

Group	Days 1-3	Day 4	Day 5 (choice phase)	n
Pre-exposed	1M suc + 25µM nicotine	1M suc	1M suc vs 1M suc + 25µM nicotine	21
Naive	1M suc	1M suc	1M suc vs 1M suc + 25µM nicotine	17
No choice	1M suc + 25µM nicotine	1M suc	1M suc vs 1M suc	27

Experiment 4b

Group	Days 1-3	Day 4	Day 5 (choice phase)	n
Arecoline	1M suc + 10µM arecoline	1M suc	1M suc vs 1M suc + 10µM arecoline	28
Caffeine	1M suc + 100µM caffeine	1M suc	1M suc vs 1M suc + 100µM caffeine	29
TMX	1M suc + 35nM TMX	1M suc	1M suc vs 1M suc + 35nM TMX	32
Scopolamine	1M suc + 80µM scopolamine	1M suc	1M suc vs 1M suc + 80µM scopolamine	32

solution variable. Models for the choice treatments ('pre-exposed' and 'naive') tested for a `Solution * Treatment` interaction, whereas the models for the 'no choice' treatment tested only for a main effect of `Solution` as a negative control. Experiment 4b was not designed factorially. To analyse these data, the treatment groups were assigned as per the drug identity but the choice solutions were designated 'sucrose' or 'drug'. We allowed the effect of solution to vary by treatment using the interaction term, and analysed the effect of solution nested within each group via within-treatment comparisons.

Model fit was assessed by plotting the results of `simulateResiduals()` and inspecting the output of the `testDispersion()` and `testZeroInflation()` functions from the `DHARMa` package (Hartig 2022). Predictor significance was tested using the `anova()` function, and non-significant interaction terms were removed. To make the pre-planned comparisons of the rates and final amounts of feeding on each choice solution within each treatment, we computed the estimated marginal means in the full models with `emmeans` (Lenth 2025) and then ran pairwise contrasts of solution–within levels of treatment, in the choice groups, i.e. `emmeans(mod, ~ Solution|Treatment)`. These only comprised one comparison per treatment group, so a custom adjustment for multiple comparisons was applied at the end of each experiment using `p.adjust(all_contrasts, method = "fdr")`.

Binned data

Feeding events were categorised by 4 h time bin, and the total amount of time feeding and number of feeding events during each time bin was summarised for each individual. Across the population these data were overdispersed, with a relatively high proportion of 0s. Given the variance structure, we fitted glmmTMBs (Brooks et al. 2017, McGillycuddy et al. 2025) with a `tweedie()` distribution for each behavioural metric and treatment group. These modelled the amount of the relevant feeding behaviour (`beh`) as a function of an interaction between solution and time bin as a factor, with a random effect of bee ID to account for repeated measures: `glmmTMB(beh ~ Solution*as.factor(time_bin) + (1|bee_ID), family = tweedie())`. Model fit was assessed in the same way as for the mixed models of the cumulative data, by plotting the results of `simulateResiduals()` and inspecting the output of the `testDispersion()` and `testZeroInflation()` functions from the `DHARMa` package (Hartig 2022). Parameter significance in these models was assessed with `car::Anova()` (Fox & Weisberg 2019) and the interaction removed where necessary. We investigated pairwise behavioural differences between the solutions at each time bin using `emmeans`, i.e. `emmeans(interaction_model, ~ Solution | time_bin)` and then made bonferroni-corrected comparisons using its `contrast()` function.

First bout duration

The first bouts that individual bees made on each solution were identified. Bees that did not make recorded feeds on both solutions were excluded from this analysis. The difference in the duration of the first bout (FBD) each individual bee, i, made on either solution was calculated:

$$\Delta\text{FBD}_i = \text{FBD}_{X,i} - \text{FBD}_{S,i}$$

where X represents the focal solution and S sucrose (or S2, in the no choice treatment).

These values were assessed for normality with Shapiro-Wilk tests and histograms, and homogeneity of variance with Levene's test. A linear model was then made (`lm(diff_firstbout ~ Treatment)`) and the significance of the predictor tested with `anova()`. To test specifically for differences in group means against 0, we used `emmeans` to get the estimated marginal mean for each treatment group and then tested these using `null = 0` in the `test()` function with an fdr correction where necessary.

PIs

Preference indices (PIs) were calculated using feeding duration and mass change data as described in the previous chapter. To test for differences in these values against 0, we fitted a linear mixed-effects model (LME) for each experiment with bee ID as a random effect as follows: `lmer(PI ~ treatment * PI_type + (1|bee_ID)`. Models with an insignificant interaction term were then re-run without it. Goodness of fit was verified by visual inspection of residual plots, and we tested parameter significance using the `anova()` function from the `lmerTest` package (Kuznetsova et al. 2017). We then compared the estimated marginal means to 0 for each PI within each group using *post hoc* fdr-adjusted contrasts: `contrast(emm, method="eff", adj="fdr")`.

Individual trajectories

To track the direction of cumulative decision making by individuals over the time bins (t), as a proportion of their total feeding, for each bee we calculated and plotted:

$$\text{PI}_{duration}(t) = \frac{\text{Cumul. }_X(t) - \text{Cumul. }_S(t)}{\text{End total }_X + \text{End total }_S}$$

where Cumul. $_X(t)$ is the cumulative amount of the focal test solution, X, drunk up to and including the end of the time bin t.

Viewable in the supplementary information are two additional sets of PI trajectories. The first expressed preference on a more absolute scale, normalised by the cumulative feeding total:

$$\text{PI}_{duration}(t) = \frac{\text{Cumul. }_X(t) - \text{Cumul. }_S(t)}{\text{Cumul. }_X(t) + \text{Cumul. }_S(t)}$$

and we also investigated the net extent of preferential feeding on the focal solution ('preference vector', PV):

$$\text{PV}_{duration}(t) = \text{Cumul. }_X(t) - \text{Cumul. }_S(t)$$

The vast majority of bees made proboscis contact with the food totalling several minutes across the recording period, but a small number made only a few seconds of detected feeding. These individuals were excluded from the visualisations using a 10 s threshold. Average lines were plotted from the mean across the time bins and smoothed using the `geom_smooth()` function.

RESULTS

Bees initially consumed nicotine at a faster rate than sucrose

In experiment 4a, bees in the no choice group fed evenly from the two randomly designated sucrose feeders (Fig. 2A). On average, individual bees within this group took just under 8 h to complete 50% of their total feeding as measured by duration (Fig. 2Ai) and number of feeding events (Fig. 2Aii). The frequency of feeds and the time spent feeding were both greatest during the first 2 h of the experiment.

No significant differences were detected by solution in the total extent of feeding at the end of the experiment in this group, whether by duration (Tables 2 and S1a) or number of feeding attempts (Tables 2 and S1b). Bees also fed on them at similar rates, as measured by time to reach 50% of total feeding (T50), (Table 2; Table S1c, Table S1d).

Similar to the bees in the no choice group, bees in both of the choice treatments tended to have fed from each food source for a similar total duration by the end of the monitoring period (Fig. 2Bi and 2Ci; linear mixed effects model (LME), Solution: $p = 0.171$, Table S2a; Table 2) and made a similar total number of feeds on each (Fig. 2Bii and 2Cii; LME, Solution: $p = 0.543$, Table S2b; Table 2) regardless of pre-exposure treatment (Solution $\times$ Treatment, Tables S2a, S2b). However, 50% of the total nicotine uptake by feeding duration was reached significantly sooner (Fig. 2Bi and 2Ci; lreg, Solution: $p < 0.001$, Table S2c; Table 2) and feeding events also initially also occurred at a faster rate (Figures 2Bii and 2Cii; lreg, Solution: $p = 0.002$, Table S2d; Table 2) such that 50% consumption was reached at around 5 and 11 h for nicotine and sucrose respectively. This was not pre-exposure-dependent overall (Tables S2c, S2d) despite the initial increase in visitation rate to the nicotine feeder being more pronounced in the drug pre-exposed bees (Fig. 2Bii vs 2Cii; $\text{T50}_{\text{feeds}}$ pre-exposed: $p = 0.054$ vs $\text{T50}_{\text{feeds}}$ naive: $p = 0.200$; Table 2).

Table 2 | Pairwise emmeans comparisons, $X - S$, of the observed differences in the extent ('total') and rate ('T50') of each feeding behaviour – either number of seconds spent feeding (duration) or counts of feeding events (feeds) – between the solutions in experiment 4a, where the focal solution (X) = S1 (no choice group) or N (pre-exposed and naive groups). T50 estimates show the pairwise difference in time during the experiment, in minutes.

No ch.	Metric	Est. ±SE	df	t	p	p(adj)
Total	Duration	12.2±27.4	25	0.445	0.660	0.660
Total	Feeds	2.66±4.53	24.8	0.588	0.562	0.660
T50	Duration	74.2±102	25	0.728	0.473	0.660
T50	Feeds	53.7±84.5	24.5	0.635	0.531	0.660
Pre-exp	Metric	Est. ±SE	df	t	p	p(adj)
Total	Duration	41.4±28.3	35.2	1.47	0.152	0.364
Total	Feeds	4.09±3.12	34.4	1.31	0.199	0.398
T50	Duration	-349±102	35.5	-3.41	0.002**	0.019 *
T50	Feeds	-281±108	35.5	-2.60	0.014*	0.054 .
Naive	Metric	Est. ±SE	df	t	p	p(adj)
Total	Duration	14.0±31.1	34.5	0.451	0.655	0.660
Total	Feeds	-1.77±3.42	33.8	-0.518	0.608	0.660
T50	Duration	-345±113	34.8	-3.06	0.004**	0.025 *
T50	Feeds	-226±119	34.8	-1.89	0.067.	0.200

To investigate the rate of feeding on either solution with a little more temporal resolution, we analysed the duration of feeding (Fig. 3a-c) and number of feeding events (Fig. 3d-f) made by bees in each treatment across a set of 4 h time bins.

In the no choice group, the mean duration spent consuming each randomly designated solu-

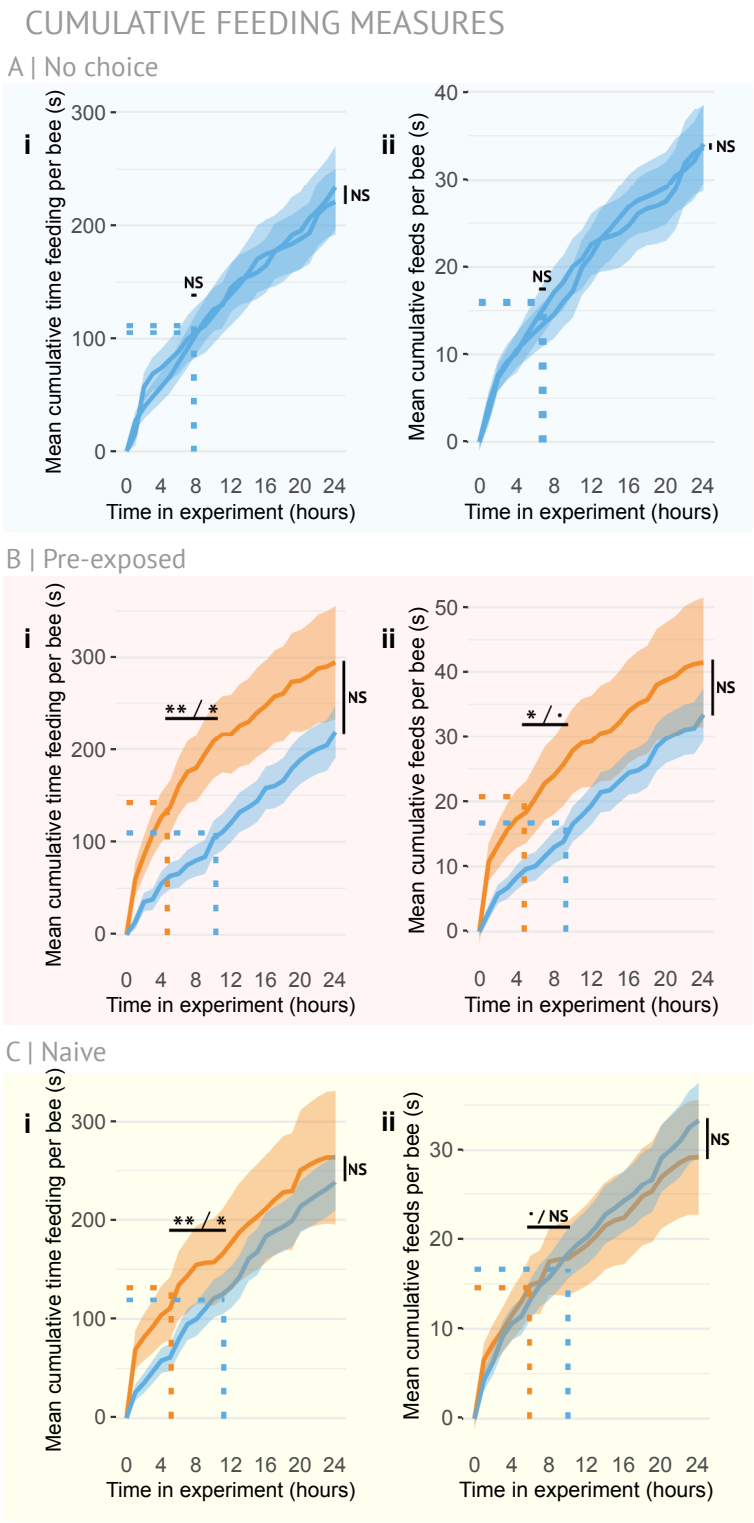


Figure 2 | **Cumulative feeding measures in experiment 4a**. **Ai–ii**: no-choice negative control ("No choice", blue background); **Bi–ii**: choice following nicotine pre-exposure and withdrawal ("Pre-exposed", red background); **Ci–ii**: choice without prior experience ("Naive", yellow background). Plots show the mean cumulative amount of (i) time spent feeding (left column), and (ii) recorded feeding events (right) per bee across a set of 1 h time bins. Lines correspond to the mean and the coloured ribbons to the 95% Monte Carlo bootstrapped cumulative confidence intervals for each solution by treatment (1 M sucrose + 25 µM nicotine shown in orange, 1 M sucrose in blue). n = 27 (no choice), 21 (pre-exposed), 17 (naive). "NS" = not significant; · = $p < 0.1$, * = $p < 0.05$, ** = $p < 0.01$. Where two symbols are given separated by a slash, significance differed before and after p-adjustment.

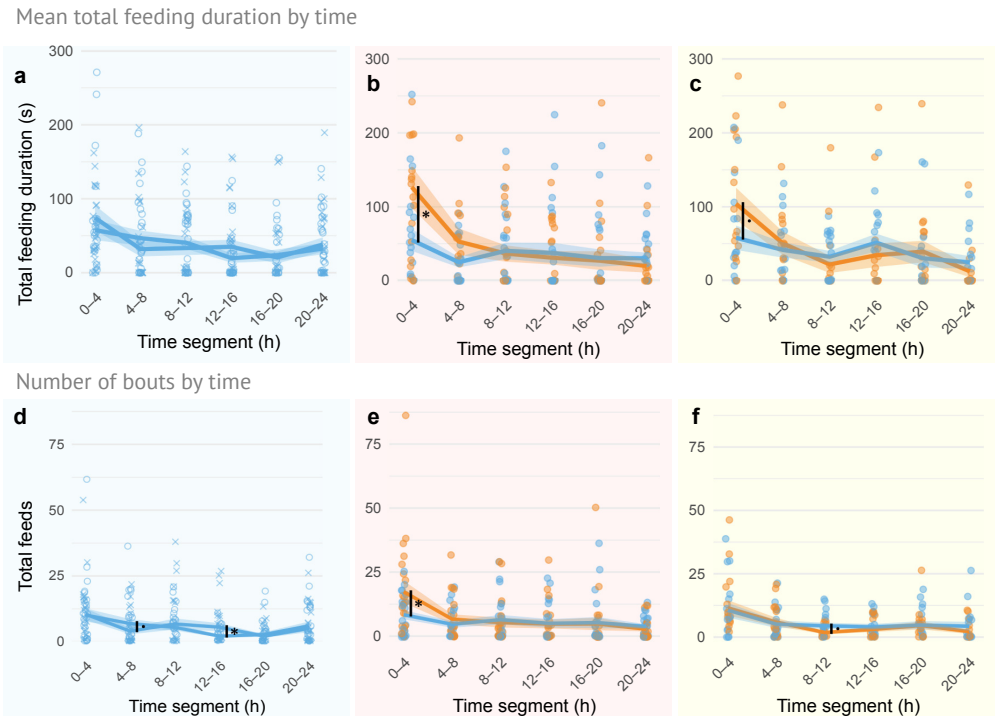


Figure 3 | **Rates of feeding by time segment in experiment 4a**. (**a-c**) Feeding duration and (**d-f**) number of feeding bouts per bee for each of the consecutive 4 hour time bins in the no choice (**a** & **d**), pre-exposed (**b** & **e**) and naive (**c** & **f**) groups. Points show observed values per bee; the line illustrates the mean ±SE (shaded ribbons). Coloured backgrounds indicate the treatment group (left: no choice, blue; middle: pre-exposed, red; right: naive, yellow). · = $p < 0.1$, * = $p < 0.05$.

tion did not differ across the time bins (Fig. 3a; Solution: $p = 0.800$, Table S3a; Table S3b). However, chance differences may have emerged in the total number of feeds recorded on the random focal solution at hours 4-8 ($p = 0.092$) and 12-16 ($p = 0.031$; Fig. 3d; Table S3d).

Bees that had been pre-exposed to nicotine did not feed significantly more on the nicotine-laced solution throughout the experiment (Fig. 3b and 3e; Tables S4a, S4c) but did feed on it for a longer average duration in the first time bin (Fig. 3b; $p = 0.014$, table S4b), and feeding visits to the nicotine solution were also significantly more numerous in this time period (Fig. 3e; $p = 0.016$, Table S4d). After this initial burst of nicotine feeding, feeding rates on both solutions stabilised and remained approximately equal for the rest of the experiment.

Bees that were naive to nicotine also tended to spend more time feeding on it in the first 4 h (Fig. 3c), but this result was marginal ($p = 0.092$, Tables S5a and S5b) and not accompanied by a concurrent increase in the amount of visits to the feeder (Fig. 3f, $p = 0.560$, Tables S5c and S5d). They then tended to feed slightly less frequently on it in the middle of the experiment such that they made marginally fewer visits to this feeder at 8-12 h ($p = 0.064$, Table S5d). Feeds by solution were otherwise largely equal throughout.

First bout durations were equal

Despite the faster rate of nicotine self-administration early in the experiment, bees from all treatment groups made first bouts of equal duration on average on the two solutions (Fig. 4; ANOVA, Treatment: $F_{2,55} = 0.316, p = 0.730$; Tables S6a and S6b; Table 3).

Table 3 | Estimated marginal means and significance tests by treatment group, assessing if the difference in first bout duration between solutions differs from zero

Group	ΔFBD ±SE (s)	df	t	p
No choice	-3.67 ± 8.69	55	-0.422	0.675
Pre-exp.	3.49 ± 9.56	55	0.366	0.716
Naive	-7.41 ± 10.4	55	-0.711	0.480

Continuous population-level PI tracking was robust to variance and revealed a transitory preference

At a population level the cumulative ratio of feeding between the two randomly-assigned sucrose solutions stabilised at 0 after about 8 h (Fig. 5a, left). However, at an individual level bees in this treatment did not forage equally between them but biased their feeding randomly to varying extents (Fig. 5a, transparent boxplot).

Bees in both choice groups expressed a preferential feeding ratio for the nicotine-laced solution throughout, which was greater following the

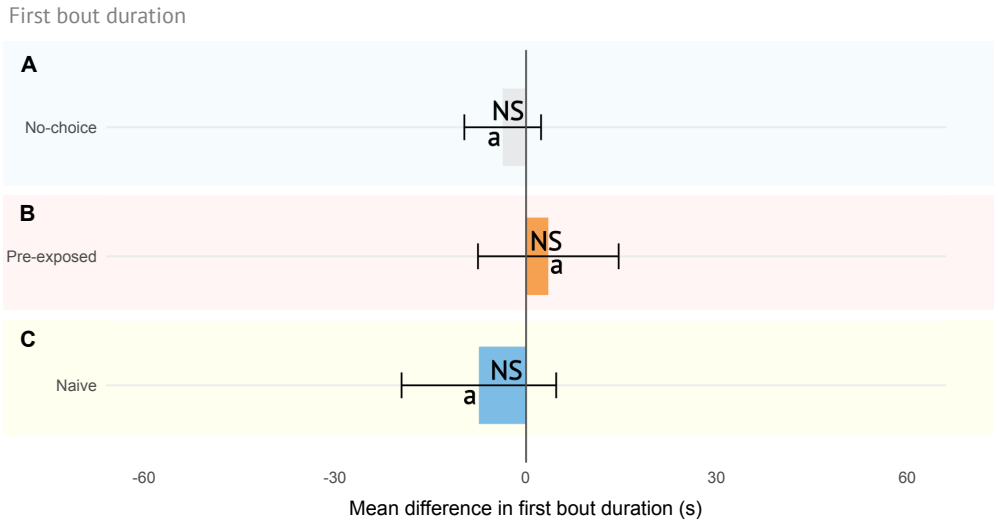


Figure 4 | **First bout duration did not differ by solution type in experiment 4a**. No significant differences in the duration of initial feeding bouts made by each bee on each choice solution were detected across or within the groups. Only data from bees that sampled from both feeders were analysed. n = 23 (no choice), 19 (pre-exposed), 16 (naive). Bars show the mean change in the duration of the first bout when feeding on the focal solution (S1 in group A, or the nicotine-laced solution in groups B and C) ± SE. Bar colour in the choice groups indicates the direction of the mean difference in FBD: either towards longer bouts on nicotine (orange) or sucrose (blue). 'NS' = 'non-significant' *post hoc* emmeans test of the group difference vs 0.

pre-exposure treatment. Pre-exposed bees moderately preferred nicotine from 0-10 h, expressing a group-level PI of about 0.4, and then gradually this ratio began to fall with further feeds down to ∼0.2 at 24 h. The naive bees also initially preferred the nicotine solution but this preference was less stable and had declined to about 0.1 at around 16 h. This slight preferential ratio was maintained thereafter. In both cases, total sucrose feeding at a population level never outweighed nicotine feeding except stochastically at the beginning.

These population-level signals emerged despite considerable inter-individual variation in both duration and mass-based preference indices (Fig. 5c-f, transparent boxplots), which consistently spanned the full range (-1 to 1) across treatment groups. Both types of preference index from bees in the naive group had noticeably smaller interquartile ranges. No significant treatment-level preferences were detected in these data, nor did these measurements vary by the type of behavioural information used to calculate them (LME, Table 4; Table 5).

Table 4 | Parameter significance F-tests with Satterthwaite degrees of freedom from linear mixed effects model of individual preference indices across all groups

Parameter	Num DF	Den DF	F	*p*
`Type`	1	62	0.611	0.438
`Treat`	2	62	0.186	0.831
`Type*treat`	2	62	1.74	0.183

Table 5 | End preference index estimated marginal mean comparisons against 0

No choice	Mean	*t*	df	*p*	*p*(adj)
Duration	-0.111	-1.03	123	0.305	0.900
Mass	0.199	1.85	123	0.066 .	0.397
Pre-exp.	Mean	*t*	df	*p*	*p*(adj)
Duration	0.016	0.134	124	0.894	0.900
Mass	-0.015	-0.130	124	0.897	0.900
Naive	Mean	*t*	df	*p*	*p*(adj)
Duration	-0.016	-0.126	124	0.900	0.900
Mass	-0.073	-0.571	124	0.569	0.900

Inter-individual variability

Some bees foraged roughly equally between identical sucrose test solutions. More commonly, they tended to form stochastic preferences that seemed to vary uniformly in magnitude (Fig. 6a).

Individual responses in the choice groups may have been less random (Figs. 6b-c). In both, the modal initial response was slight to moderate nicotine preference. Pre-exposed bees mostly developed moderate to strong nicotine preferences (∼0.5 - 0.75), some fed roughly evenly between the solutions, and another subset proportionally reduced their nicotine intake over time down to a ratio indicating moderate aversion (∼ -0.5; Fig. 6b). Response trajectories in the naive bees stratified in a similar though perhaps slightly narrower way. Approximately half acquired slight to moderate preferences for the drug, and the others responded with mild to moderate avoidance.

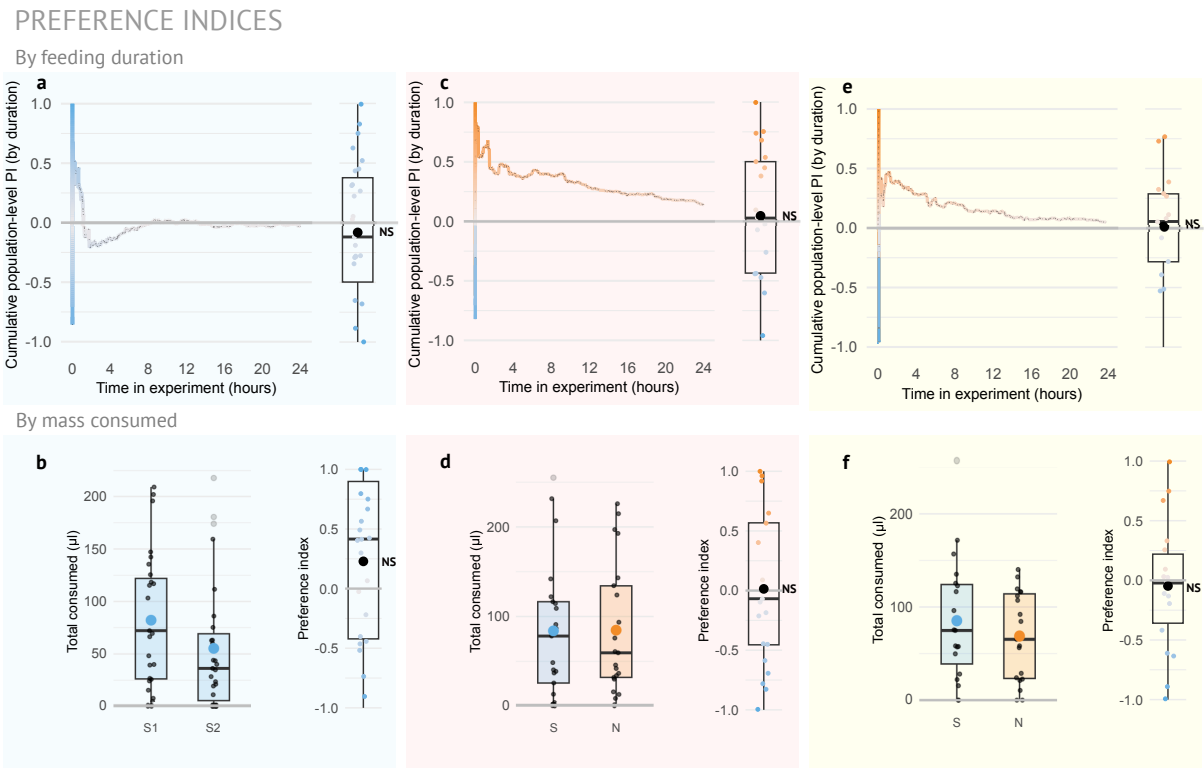


Figure 5 | **Comparison of preference index metrics in experiment 4a.** (**a-b**) No choice, (**c-d**) nicotine pre-exposed, (**e-f**) nicotine naive. Line graphs in the top row show the group-level preference calculated cumulatively with each feeding event in time across the population of bees using the duration data. The accompanying boxplots in this row illustrate the distribution of duration-based preference indices calculated on an individual basis at 24h. The shaded boxplots below show the mass consumed of each solution per bee, and the unshaded boxplots to their right express the distribution of individual mass-based preference indices calculated from these data. 'NS' denotes statistically insignificant comparison of the estimated marginal mean of the preference index vs 0.

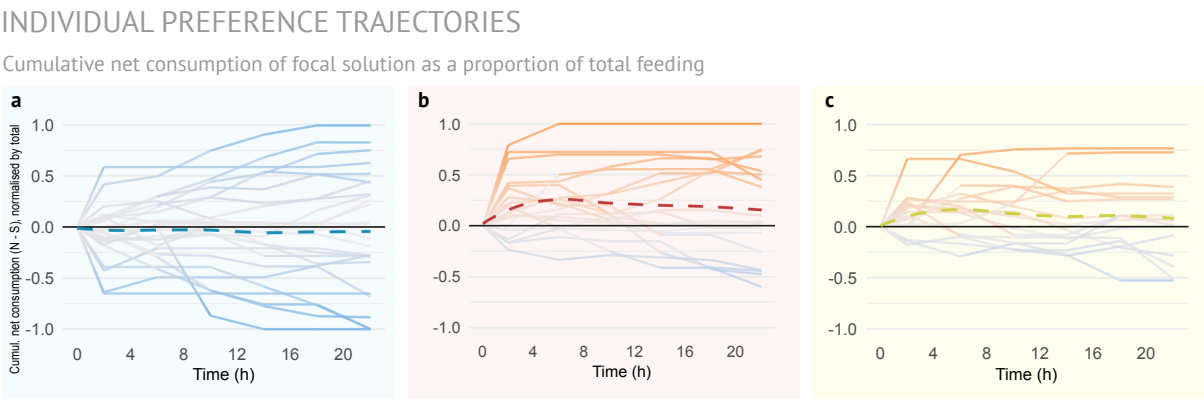


Figure 6 | **Inter-individual behavioural variation in experiment 4a**. Duration-based preference index trajectories across 4h time bins of bees in the (**a**) no choice, (**b**) nicotine pre-exposed and (**c**) nicotine-naive treatment groups. Lines show the cumulative sum of the focal solution consumed ('sucrose 1', left; or 1M sucrose + 25uM nicotine, middle and right) minus that of the other solution ('sucrose 2', left; or 1M sucrose, middle and right), normalised by the total of all food consumed at the end of the experiment (S1+S2 or N+S at t = 24). Data were filtered to exclude 4 bees that consumed food in very small quantities using a 10 second threshold. n = 26 (no choice), 19 (pre-exposed), 16 (naive).

OTHER DRUGS (experiment 4b)

Bees may have showed an initial arecoline bias

Feeding differences by choice solution were less pronounced in this experiment. Neither treatment type nor drug presence predicted the extent of feeding at 24 h, as measured by cumulative duration (left plots in Fig. 7 facets a-d; Table S8a) or cumulative number of feeding events (Fig. 7a-d, right; Table S8b), and interactions were not detected between the two variables (Tables S8a, S8b). Accordingly, planned within-treatment comparisons of the difference in these metrics between the solutions were not significant (Table 6).

Bees in most groups took longer on average to reach 50% of their total feeding duration on the drug-laced solution, except those in the arecoline treatment (Table 7): similar as observed with nicotine in experiment 4a, bees in this group tended to reach 50% of the total feeding duration on this solution earlier than for the non-drugged alternative option. This finding was marginal, however, (Table 7) and no significant overall interactions or treatment effects were detected in the rate data (Tables S8c and S8d).

Table 6 | Pairwise comparisons of the total duration of feeding on the focal solution relative to sucrose ('duration'), and of the number of bouts on each ('feeds'). Data were square root transformed therefore estimates and SEs are shown on this scale.

Arecoline	Est.±SE(sqrt)	df	t	p	p(fdr)
Duration	1.04±1.73	114	0.604	0.547	0.633
Feeds	0.614±0.601	111	1.02	0.310	0.508
Scopol.	Est.±SE(sqrt)	df	t	p	p(fdr)
Duration	-0.581±1.58	109	-0.367	0.714	0.762
Feeds	-0.325±0.548	107	-0.594	0.554	0.633
TMX	Est.±SE(sqrt)	df	t	p	p(fdr)
Duration	-1.69±1.64	117	-1.04	0.303	0.508
Feeds	-0.876±0.572	115	-1.53	0.128	0.410
Caffeine	Est.±SE(sqrt)	df	t	p	p(fdr)
Duration	2.24±1.71	116	1.30	0.195	0.508
Feeds	0.653±0.598	113	1.09	0.277	0.508

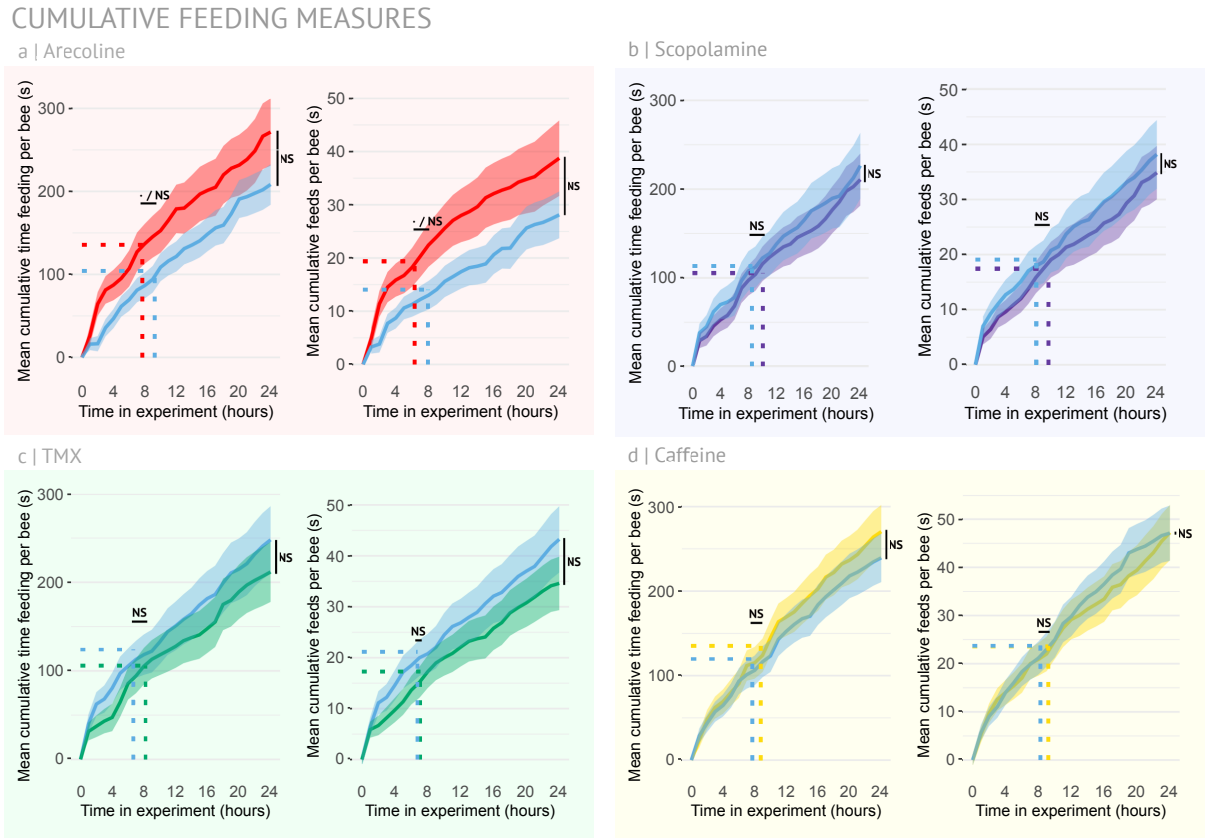


Figure 7 | **Mean per bee cumulative feeding choices by group and behavioural measure in experiment 4b**. **Ai-ii**: 10µM arecoline (red); **Bi-ii**: 80µM scopolamine (purple); **Ci-ii**: 35nM TMX (green); **Di,ii**: 100µM caffeine (yellow). The average amount of feeding per bee according to each metric (duration and number of recorded feeding events – plots 1 and 2 respectively within each facet). The line corresponds to the mean and the coloured ribbons to the 95% Monte Carlo bootstrapped cumulative confidence intervals for each solution by treatment, where 1M sucrose is shown in blue). n = 28 (arecoline), 29 (caffeine), 32 (TMX), 32 (scopolamine). "NS" = not significant; · = $p < 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 7 | Pairwise emmeans comparisons of the time difference in minutes at which the T50 point was reached for the drug relative to sucrose ($Midpoint_{-drug} - Midpoint_{-suc}$) for each behavioural measure of feeding.

Arecoline	Δt ±SE (mins)	df	t	p	p(fdr)
Duration	-161±95.7	114	-1.68	0.095 .	0.410
Feeds	-152±91.4	114	-1.66	0.099 .	0.410
Scopol.	Δt ±SE (mins)	df	t	p	p(fdr)
Duration	76.8±87.6	109	0.877	0.383	0.510
Feeds	80.1±83.7	109	0.957	0.341	0.508
TMX	Δt ±SE (mins)	df	t	p	p(fdr)
Duration	85.2±90.6	117	0.940	0.349	0.508
Feeds	6.52±86.5	117	0.075	0.940	0.940
Caffeine	Δt ±SE (mins)	df	t	p	p(fdr)
Duration	149±94.8	116	1.57	0.120	0.410
Feeds	147±90.7	116	1.62	0.108	0.410

We analysed the rate of feeding with some more temporal resolution by time bin (Fig. 8). Bees did not feed for significantly more time on arecoline than sucrose overall (Fig. 8ai; glmmTMB, Solution: $p = 0.151$; Table S9a) though may have done in the final 4 h (p = 0.074; Table S9b). Bees did make significantly more detected feeding bouts on this arecoline-laced solution throughout, however (Solution: $p = 0.028$; Fig. 8aii; Table S9c), particularly in the first time bin ($p = 0.031$; Table S9d).

The other drugs were less influential on choice behaviour through time. Scopolamine had no global effect on the amount of feeding at either feeder by either metric, nor during any particular time segment (Fig. 8bi and 8bii; Tables S10a-d). TMX did not affect the feeding duration on either solution overall or between time bins (Fig. 8ci and 8cii; Tables S11a, S11b), but marginally reduced the number of detected feeding events made throughout the monitoring period (Solution: $p = 0.083$; Table S11c). This effect was most salient in the first 4 h ($p = 0.084$; Table S11d).

Caffeine did not modulate feeding duration (Fig. 8di; Table S12a-b) or number of feeding bouts (Fig. 8dii; Table S12c-d) through the experiment except, potentially, in the number of feeding bouts made towards the end ($p = 0.036$; Table S12d).

First bout durations were no different by solution

Bees made initial feeding bouts on either solution that were equal in duration across treatments (Fig. 9; ANOVA, $F_{3,104} = 1.36, p = 0.260$, Tables S13a-b), with the possible exception of scopolamine (Table S13a). None differed significantly from 0 (Table 8).

Population-level preferences

In the first two to three hours, bees in the arecoline group established a strong population-level

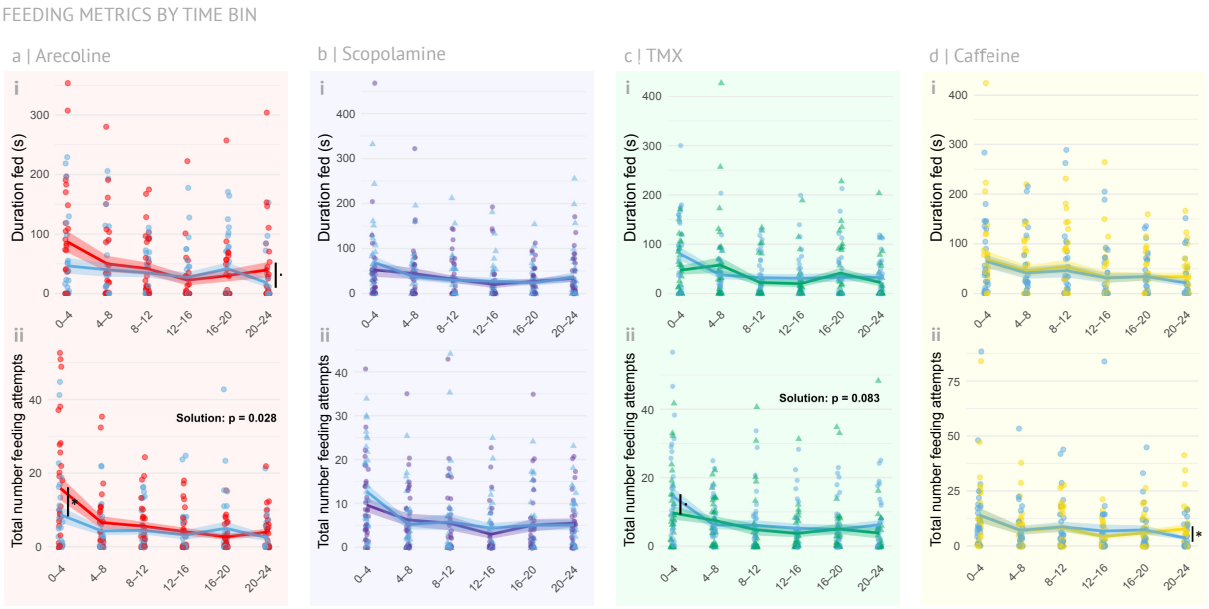


Figure 8 | **Rates of feeding by time segment in experiment 4b**. (**i**) Feeding duration and (**ii**) number of feeding bouts made by each bee on each solution during each of the consecutive 4 hour-wide time bins. Points show observed values per bee; the line illustrates the mean ±SE (shaded ribbons). Coloured backgrounds indicate the treatment group (column **a**: arecoline, red (n = 28); column **b**: scopolamine, purple (n = 32); column **c**: TMX, green (n = 32); column **d**: caffeine, yellow (n = 29). Line colours indicate the identity of the solution, with 1M sucrose shown in blue.

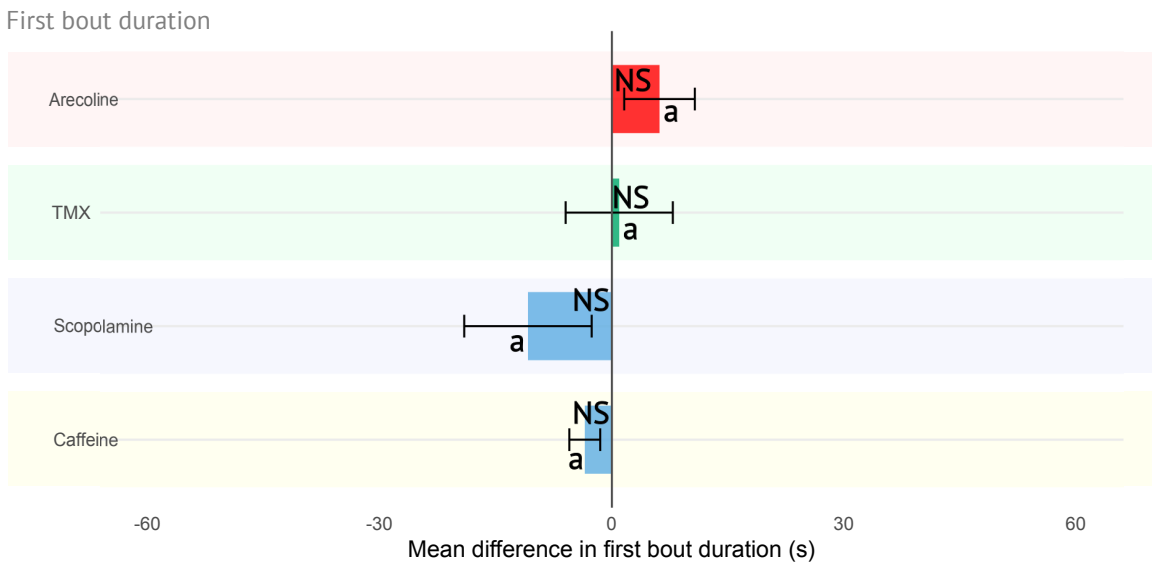


Figure 9 | **First bout duration was again no different by solution in experiment 4b**. Initial feeding bouts made by each bee on each choice solution were no different in duration on average within or across treatment groups. Only data from bees that sampled from both feeders were analysed. Bars show the mean difference in first bout duration between the solutions across bees in the treatment group, ±SE, with positive values denoting longer bouts on the focal solution (though these differences were insignificant). The colour corresponds to the identity of the solution that produced longer feeding bouts on average, with blue being the undrugged sucrose. From top to bottom: 10µM Arecoline (n = 25); 35nM TMX (n = 27); 80µM scopolamine (n = 31); 100µM caffeine (n = 25). NS = 'non-significantly different from 0'; lettering denotes lack of differences between groups.

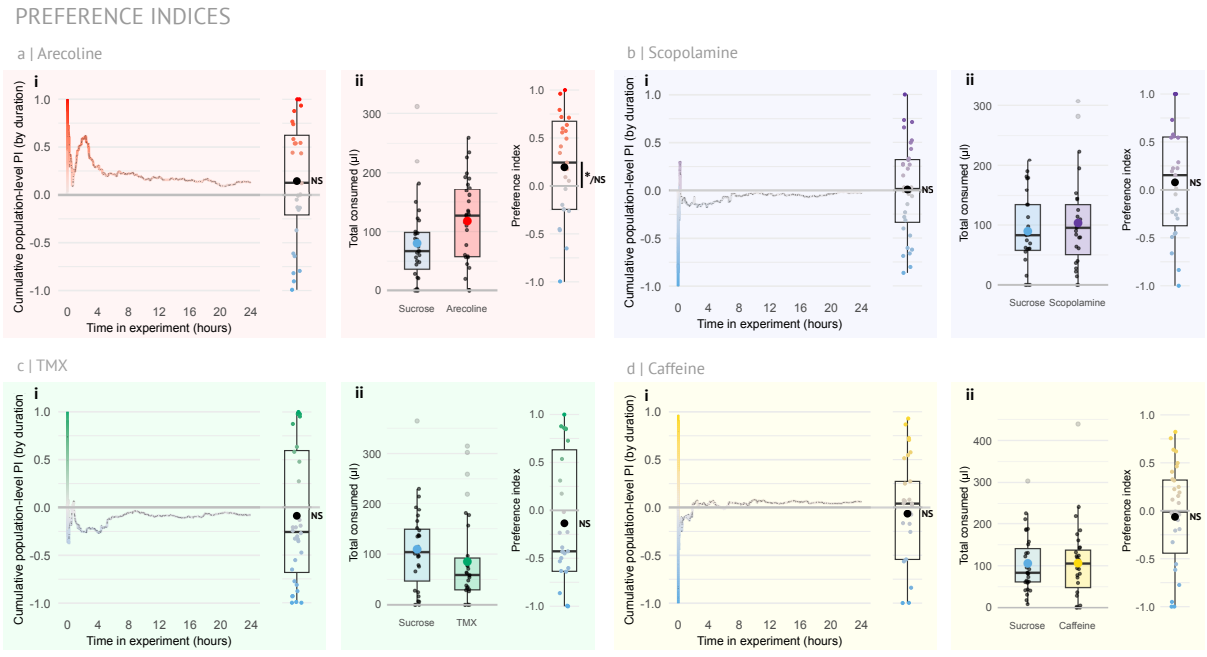


Figure 10 | **Comparison of preference index metrics in experiment 4b**. (**i**) Continuous traces show the group-level preference calculated cumulatively with each feeding event in time across the population of bees using the feeding duration data. The boxplot accompanying each line graph illustrates the distribution of preference indices calculated on an individual basis using the feeding duration data at the end of the experiment. (**ii**) The shaded boxplots in the facets to the right illustrate the mass consumed of each solution per bee, and to the right of these are the distributions of the per individual mass-based preference indices calculated using these data (unshaded boxplots, right). Large dots show the mean. Significance denotes the result of estimated marginal mean comparisons against 0 (NS = 'non-significant', * = $p < 0.05$). (**ai-ii**) arecoline (n = 28 duration, 31 mass), (**bi-ii**) scopolamine (n = 32 duration, 27 mass), (**ci-ii**) TMX (n = 32 duration, 31 mass), (**di-ii**) caffeine (n = 29 duration, 31 mass).

Table 8 | Group emmeans tests vs 0 of the difference in first bout duration between the solutions

Group	ΔFBD ±SE (s)	df	t	p	p(adj)
Arecoline	6.14 ± 6.54	104	0.939	0.350	0.699
Scopol.	-10.8 ± 5.87	104	-1.84	0.068 .	0.273
TMX	0.948 ± 6.29	104	0.151	0.881	0.881
Caffeine	-3.50 ± 6.54	104	-0.535	0.594	0.792

preference for the drug that climbed to 0.6 before dropping to around 0.25 at 4 h (Fig. 10ai). From there this preferential feeding ratio diminished slightly but was mostly maintained until the end of the experiment. The accompanying boxplot of individual feeding duration-derived preference indices in Fig. 10ai shows an interesting response distribution, with a cluster of individuals expressing a strong preference for the drug at 24 h (0.5-1); another, smaller group largely having avoided it (-0.5 to -1); and some in the middle who showed little bias either way. The mass change measurements also illustrate that bees tended on average to consume higher quantities of the drugged solution, with a clear accumulation of PI values at the upper end of the preference index range (Fig. 10aii) creating a significant preference before adjustment (Table 10).

The preference traces for TMX and scopolamine reveal a degree of avoidance at the beginning of the experiment which set in early but not immediately and peaked at about 3-4 hours. This was then overcome to some extent as the trajectories began to converge back up towards 0 (Fig. 10bi) and -0.1 (Fig. 10ci) at about 8 hours in, after which point these population-level feeding ratios were largely maintained. The skewed TMX boxplots indicate that most bees had started to avoid the TMX-laced solution although a small subset of individuals consumed it in unusually large quantities, possibly showing strong preferences for it (Fig 10cii).

Caffeine was slightly preferred at a population level from 2 h at a consistent ratio of just under 0.1. Few patterns were visible in the boxplots: PIs of individual subjects derived at the end from each behavioural measure appeared to be pretty evenly distributed across the range.

Overall, individual PIs at 24 h did not vary by treatment or by the behavioural metric used to calculate them (LME, Table 9; Fig. 10 transparent boxplots). They did not differ significantly from 0 either, with the possible exception of an arecoline preference calculated from the mass change data (Table 10).

Table 9 | Parameter significance F-tests with Satterthwaite degrees of freedom from linear mixed effects model of preference indices

Parameter	Num DF	Den DF	F	p
`Type`	1	112	1.00	0.318
`Treat`	3	117	1.33	0.267
`Type*treat`	3	112	0.780	0.508

Table 10 | Comparisons of preference indices at the end of the experiment vs 0, before and after fdr-adjustment

Arecoline	Mean	t	df	p	p(adj)
Duration	0.130	1.27	138	0.207	0.578
Mass	0.218	2.13	138	0.035 *	0.283
Scopol.	Mean	t	df	p	p(adj)
Duration	-0.007	-0.072	137	0.943	0.943
Mass	0.052	0.515	147	0.607	0.694
TMX	Mean	t	df	p	p(adj)
Duration	-0.103	-1.05	137	0.296	0.578
Mass	-0.122	-1.24	139	0.217	0.578
Caffeine	Mean	t	df	p	p(adj)
Duration	-0.080	-0.785	138	0.434	0.578
Mass	-0.089	-0.874	138	0.384	0.578

Individual responding

Responses in the arecoline individual preference trajectories at the end of experiment 4b were relatively dense in the 0.5–1 range (Fig. 11a). This perhaps indicates that it is possible certain individuals could have preferred the drug quite strongly. Though bees expressing preference and avoidance for the drug were similar in number, the net extent of arecoline feeding tended to be greater (Fig. S14, top left).

Bees in the TMX treatment group mostly avoided the drug, but a notable subset could have biased their feeding towards it (Fig. 11c and S14). Compared to the nAChR agonists, the scopolamine and caffeine response distributions were perhaps slightly more narrow and even (Fig. S14).

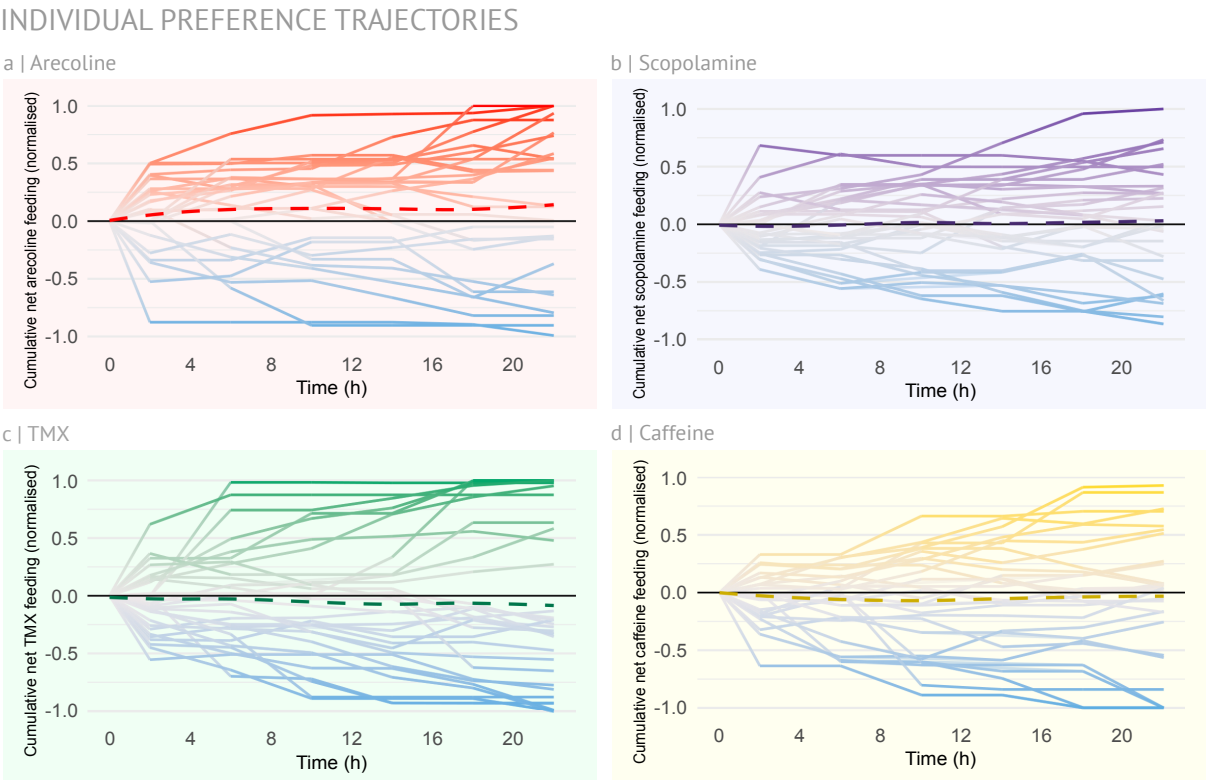


Figure 11 | **Inter-individual behavioural variation in experiment 4b**. Duration-based preference index trajectories across 4h time bins of bees in the (**a**) arecoline, (**b**) scopolamine, (**c**) TMX and (**d**) caffeine treatment groups. Lines show the cumulative bias towards or away from the drugged solution as a proportion of the individual's feeding at the end of the experiment. This was calculated as the cumulative sum of the focal solution consumed (1M sucrose + either 10µM arecoline (top left), 80µM scopolamine (top right), 35nM TMX (bottom left) or 100µM caffeine (bottom right)) minus that of the undrugged 1M sucrose; normalised by the sum total of all food consumed at the end of the experiment (X+S at t = 24). Data were filtered to exclude 3 bees that consumed food in unusually small quantities using a 10 second threshold. n = 28 (arecoline), 30 (scopolamine), 32 (TMX), 28 (caffeine).

DISCUSSION

Ours is the first direct exploration of the development of preferences in bees for food laced with pharmacologically active compounds. The experiments were designed to examine how pre-exposure to such compounds added to food impacted the feeding decisions made by bees during a period after withdrawal. In experiment 4a, we verified that naïve bumblebees show a subtle preference for nicotine-laced food at the population level but that this effect is underpinned by a strong initial drug preference after which intake is titrated such that the measured value at 24h is attenuated. On average the magnitude of preference is stronger with previous exposure to and withdrawal from the drug, but individuals perhaps vary in their susceptibility to it. Arecoline also tended to elicit patterns of preferential feeding behaviour across the population and between subjects similar to those of nicotine. Certain individuals could have expressed strong and persistent preferences for 35nM TMX early in the experiment, but at a population level they were outnumbered by those that appeared to find it either mildly or strongly aversive. Responses to scopolamine and caffeine were less partitioned and did not seem to contain evidence of withdrawal-mediated preferences for these compounds. Our data support the view that certain nAChR agonists (such as nicotine and arecoline) could potentially produce addiction in bees.

Nicotine intake was biased towards the beginning of the monitoring period in experiment 4a

Within the first few hours of the experiment, bees from both choice groups consumed nicotine significantly faster than sucrose, reaching 50% of their individual feeding duration on nicotine significantly sooner (despite this threshold being larger). Trends in the feeding event count data were less pronounced but bees in the pre-exposed group did initially make visits to the nicotine-laced feeder at a significantly faster rate too. This initial nicotine bias was common to most individuals and stronger following pre-exposure.

By 24h, when feeding choices are aggregated within individuals and weighted equally as ratios,

preferences are not visible. However, the population traces produced using absolute food consumption revealed preferential feeding ratios in favour of nicotine. This suggests that nicotine-preferring bees may have consumed greater quantities of food as a result of their drive to obtain the drug.

First bout durations were not significantly different between the solutions, indicating that nicotine preferences were probably not driven by taste. In other plant tissues nicotine serves as a phagoinhibitory defence compound, and in 1M sucrose both honeybees and bumblebees show slightly reduced responsiveness to it at 10-100uM in 1M and 0.5M sucrose (Mustard et al. 2023, Tiedeken et al. 2014) and more dilute sucrose laced with similar concentrations of the drug does begin to reduce its uptake by free-flying honeybees (Köhler et al. 2012). Our bees did not reject 25 μM nicotine in 1 M sucrose, whether a novel food source to them or not, either because they couldn't taste it or found it insufficiently deterrent. The slight opposite tendencies in these data (for pre-exposed bees to feed for slightly longer on it and naive bees for slightly less time) may support the latter conclusion as pre-exposed bees could have experienced a conditioned taste preference. In any case, the preferential nicotine feeding we identified towards the beginning of the experiment was not immediately manifest and was probably therefore encoded instead through state-dependent post-ingestive feedback.

Probably due to state-dependent post-ingestive reinforcement

In mammals, nicotine use elicits mild euphoria. It is also thought to be rewarding to insects given that it stimulates locomotor behaviour (Bainton et al. 2000, Sanchez-Díaz et al. 2015, Zhang et al. 2016) which is believed to index its function as a positive reinforcer (Wise & Bozarth 1987) and the drug has been reported to modify bee foraging behaviour in a way that would be expected if the nectar were highly valued (Singaravelan et al. 2005, Baracchi et al. 2017). Our results seem to concur that nicotine can be hedonically reinforcing to insects, but the temporal instability of this self-administration behaviour and its increase in magnitude with pre-exposure suggests that nicotine's post-ingestive hedonic quality is state-dependent and that value assessments of drug-laced food may be ongoing.

In our assay, bees were able to interact hedonically with the drugged food *ad libitum*. Nicotine responses are biphasic such that higher doses produce aversive side effects (Koob & Moal 2006), and bees experiencing post-ingestive malaise might reasonably be predicted to reduce their voluntary exposure to the offending food source (Wright et al. 2010). This reversal of nicotine-biased decision-making after a few hours may therefore reflect the imposition of homeostatic constraints on their feeding decisions. It's possible, then, that the similar feeding rates by solution through the remainder of the experiment could be the result of active feeding decisions through which bees continued to titrate their nicotine intake. Alternatively, this might reflect the removal of pharmacological enhancement to an ongoing reevaluation process. In either case, where homeostatic mechanisms are involved, reward assessment is in flux and thus 'preference' changes as the need for the compound diminishes. This arguably relates only peripherally to natural foraging scenarios: given the very small volumes of food offered by flowers (Tiedge & Lohaus 2017) nicotine may function more unequivocally to reinforce floral reward learning. This would be a useful thing to test with future experiments.

Bees in the pre-exposed group increased their nicotine intake at the start of the monitoring period to a greater extent than the naive bees. This is what we would have predicted if nAChR expression had been upregulated as a result of the development of tolerance induced by chronic nicotine treatment (Christen et al. 2016, Wittenberg et al. 2020) because the availability of more active receptors displaces dose-response curves (Stolerman et al. 1974). It is possible that this also reflects addictive, withdrawal-induced drug-seeking – this too would increase the intensity of temporary nicotine self-administration in comparison to the naive bees, particularly if nicotine pre-exposure had blunted their natural reward sensitivity or otherwise caused them to devalue natural rewards (Peechatka et al. 2015, Volkow et al. 2010, White 1998) and thus enhanced the relative value of the nicotine-laced solution. Unlike the naive bees, individuals in this treatment group made more visits to the nicotine-laced feeder at the beginning of the experiment in addition to feeding on it for longer, which perhaps suggests that they did experience additional motivation to seek the compound and were able to act on it. Further work will be required to verify the timescales of nicotine withdrawal in bees and characterise its behavioural symptoms, and then future experimentation would be able to identify the causes of this pattern. The extent to which tolerance to nicotine-laced nectar could actually develop in the field is another important question. In any case, this potential 'bingeing' behaviour by bees on nicotine-laced nectar is a novel finding, and the fact that it is enhanced with pre-exposure hints at addictive causes.

If, as we suggest, nicotine preferences are in-

stantiated more consistently across the population within the first few hours of monitoring, it may explain why low doses of nicotine in choice tests of a few hours in duration have tended to prove attractive to bees (Singaravelan et al. 2005, Baracchi et al. 2017, Köhler et al. 2012) but when the duration is increased to 24h and/or the concentration increased, preferences are ostensibly absent (Tiedeken et al. 2014, Palmer-Young et al. 2017). Other factors may be responsible or contribute – bees in the former group were able to move freely, for example, whereas those used in the latter studies were not able to fly.

It is possible that certain bees were more susceptible to expressing nicotine preference

Among other groups of animals, differential vulnerability to drug addiction is known to be linked to initial drug sensitivity (Pomerleau 1995) which can vary by genes encoding receptor proteins (Portugal & Gould 2008, Semaan & Khan 2023, Prescott & Kendler 1999), age – adolescents generally being more sensitive to the rewarding effects of drugs and less to aversive effects (Yuan et al. 2015, Colyer-Patel et al. 2023) – sex (Faraday et al. 2005, Lenoir et al. 2015, Chaudhri et al. 2005), stress (Sinha 2008, Yap & Miczek 2008) and personality traits (Mitchell & Potenza 2014, Blanchard et al. 2009, Kuhn et al. 2019), all of which could in principle apply to bees with nicotine.

Some fragmentary evidence suggests that bees can vary behaviourally in their drug responses: Baracchi et al. (2017) mention briefly in their discussion that they observed a notable degree of variation between the responses of bees to nicotine during their learning task, and Sokolowski et al. (2012) saw that bees showed bifurcating patterns of feeding when presented with a choice to consume ethanol, with a subset of individuals increasing their voluntary drug consumption and others choosing to avoid it. Here, it is hard to say whether we found nicotine-laced food to be strongly preferred into the longer term by a subset of bees. The individual preference trajectory distributions were less symmetrical than in the no choice group, but whether they contain convincing behavioural clusters is more equivocal.

In all three studies these conclusions were reached by observation rather than statistical testing. Investigating the causes of this variation in bees might shed light on the evolutionary problem of nectar drugs but will require a different approach. In future, it might be interesting to see whether initial sensitivity to drug-laced reward corresponds to strength of preference for the drug in similar choice assays after forced pre-exposure and withdrawal, and whether these same individuals are also more susceptible to developing addictions to other drugs. The potential existence of individual differences in drug sensitivity ought to make us cautious about averaging data across subjects in future addictive studies.

In experiment 4b, arecoline seemed to produce similar patterns, with some side effects

Like nicotine, arecoline is a naturally-occurring nAChR agonist, and there were similarities in the ways bees responded to it. Arecoline produced a pattern of initial preferential feeding by number of feeds, though unlike nicotine bees went on to return to this feeder more often throughout the experiment and there were no statistically significant changes to the duration of feeding. The population-level preference index trace for arecoline produced from feeding duration data was similar in shape to that of nicotine, peaking and stabilising at similar points in time and at similar levels, and this time indicating a statistically significant sustained preference. Together this suggests that arecoline could have had addictive and similar effects on bees to nicotine, but it may have elicited more irregular patterns of feeding.

A recent piece of work tested the effects of 6 days of exposure of bumblebees to 0.1 μM or 100 μM arecoline and found that, in the high dose group, bees had become hyperactive (Liu et al. 2025). It is intriguing that in our study we saw that changes of this kind were specific only to this feeder – the change to both forms of movement seems to suggest some sort of increase to locomotion, but visitation rates in our assay weren't generally elevated across both solutions (at least not at 10 μM).

The trend in the first bout duration data seems to suggest that it could be possible that bees were getting some sort of post-ingestive feedback while consuming the solution. At a population level, bees also consumed higher volumes of the arecoline-laced solution than the undrugged sucrose, showing a significant preference for it. Liu et al. (2025) also revealed a mild though non-significant improvement to learning and memory in a PER assay by 0.1uM arecoline, and identified that it had changed expression of addiction-related genes in the Kenyon cells. These findings appear to be concordant with ours but it is difficult to offer mechanistic links because so few studies have yet investigated the affinity of arecoline for insect nAChRs and mAChRs, its ability to modulate neural circuits, and the resultant effects on these behaviours of interest. Whether or not arecoline has a nectar presence, these data support the hypothesis that sublethal doses of related drugs can induce behavioural changes potentially indicative of addiction, implying the possibility for this phenomenon to be much more taxonomi-

cally widespread.

TMX probably wasn't addictive - at least to most individuals

As a neonicotinoid – 'new' or 'synthetic nicotine-like compound' – we may have expected TMX to have similar effects on bees to nicotine and arecoline. Some literature does find that bees can acquire foraging preferences for it at low doses (Kessler et al. 2015, Arce et al. 2018) through post-ingestive feedback (Parkinson et al. 2023). We used the same concentration of TMX in food as Arce et al. (2018).

Our bees didn't show preferential TMX feeding and they did not behave very similarly to those in the nicotine and arecoline treatments. If anything, for the first few hours they appeared to bias their feeding away from the drug at a population level. TMX also tended to be visited less often throughout the experiment. This initial aversion was attenuated through time, though not fully reversed: a slightly TMX-avoidant feeding ratio was maintained from about 6 hours onwards though no overall aversive effect was detected.

Bees didn't seem to be able to respond to its presence in food during the first bout, probably because they cannot taste it (Parkinson et al. 2023), but this lack of preference was somewhat surprising. I performed a caged assay and found that naive bumblebees showed a slight but statistically insignificant preference for the drug at similar concentrations (unpublished data), so it's possible that the effect is slight and/or dose-dependent. In comparison with the literature reporting TMX preferences in bees, a much bigger body of work finds that various patterns and doses of TMX exposure impair their learning and memory processes (Mustard 2020, Stanley et al. 2015, Siviter et al. 2018, Samuelson et al. 2016, Ludicke & Nieh 2020) and it is not very clear how these two groups of findings fit together. These experiments generally make use of drugs at higher concentrations. At present, though, it is challenging to draw general conclusions about the effects of TMX because they are so dependent on factors like dose, time, timing of delivery with respect to task, and experimental context; and no one really knows enough about the distribution of TMX-sensitive nAChRs in the insect body and how these systems interact in order to make sense of their findings. More research will be required to understand at a mechanistic level why bees struggle in learning and memory assays with TMX exposure but seem sometimes to specialise on these feeders in foraging tasks. Exploring how naive bees would choose to ingest TMX in the short and long term under voluntary exposure conditions could help us begin to understand the drug's effects on learning and memory in less artificial and constrained contexts.

From the individual choice trajectories it appears most bees did not prefer food laced with TMX, but also that a small minority could possibly have biased their feeding decisions quite strongly towards it. This pattern might just be attributable to chance. Alternatively, it might indicate that TMX could have the potential to be addictive to susceptible individuals.

TMX is known to behave a bit differently at nAChRs to nicotine and arecoline: despite being a neonicotinoid, it is unusual in its low binding affinity for recognised nAChR types in bees (Nauen et al. 2003, Tan et al. 2007, Thany 2011). It is thought to exert its effects indirectly following metabolism to clothianidin (Moffat et al. 2016, Coulon et al. 2018) which is a known nAChR agonist (Brown et al. 2006, Tan et al. 2007, Nauen et al. 2003), but it seems to produce different behavioural effects (Williamson et al. 2014, Kessler et al. 2015, Moffat et al. 2016). Given that its exact mechanism of action is contested, its receptor targets across the body also remain largely unknown. Hyperactivity and locomotor changes are common effects of treating bees with field-relevant doses of neonicoitinoids, so perhaps it's possible that TMX agonises an as yet unidentified nAChR subtype and/or that it is a relatively more potent agonist of nAChRs in the VNC ganglia where ACh is used to trigger voluntary muscle contractions. Whatever the cause, these behavioural differences by compounds that ostensibly share the same mechanism of action caution that the behavioural symptoms of addiction could potentially be quite diverse. This set of findings is obviously less relevant to the evolutionary addictive hypothesis but is still ecologically pertinent given ongoing assessments of the risk sublethal pesticide exposure poses to non-target organisms, and future studies of TMX addiction could be helpful in exploring how drug dependence might develop in bees in the presence of clearer fitness costs.

Caffeine did not elicit addictive or even preferential feeding behaviour

We didn't find evidence that bees had become addicted to caffeine. Bees did not feed faster on the caffeinated solution at the beginning of the experiment, nor did their individual preference trajectories show discernable response clustering. First bouts on the caffeinated solution were no longer in duration than those on sucrose (if anything, they tended to be a bit shorter).

Chronic pre-exposure to caffeine in bees could hypothetically provoke upregulation of adenosine and dopamine receptors which may have a

homeostatic influence. In other animals, three days of oral caffeine administration is sufficient to produce these changes (Shi et al. 1994, Fredholm 1982, Johansson et al. 1997) yet the drug has only a weak ability to drive addictive self-administration behaviour compared to other psychostimulant drugs (Deneau et al. 1969, Ferré 2016, Fredholm et al. 1999) because it is less potent at engaging and dysregulating reward pathways, and animals are often deterred by its bitter taste. The fact we didn't observe addictive self-administration behaviour perhaps therefore isn't surprising. It would nonetheless be interesting to see whether pre-exposure to caffeine changes how bees might respond to it in an olfactory conditioning task in the style of the nicotine PER experiment presented in chapter 2.

Based on previous research showing preferences for caffeine-associated food sources (Arnold et al. 2021, Si et al. 2005, Jones & Agrawal 2022, Thomson et al. 2015, Singaravelan et al. 2005, Couvillon et al. 2015), we expected that caffeinated rewards would be more memorable to the animals, particularly in the longer term, due to their pharmacological influence on reward learning; and that this would manifest as a preference for the caffeinated solution that was potentially expressed more strongly towards the end of the experiment. Contrary to our predictions, when averaging across equally weighted individual mass-based and duration-based preference indices at 24h we found that bees did not prefer this solution.

Caffeinated rewards probably bias feeding decisions in bees by encouraging continued seeking of the caffeine-associated cue through the drug's pharmacological influence on memory rather than by driving caffeinated reward-seeking behaviour (Arnold et al. 2021, Wright et al. 2013). That is not to say that the resulting preferences are indelible: when bees are 'primed' to remember a floral odour blend by consuming caffeinated food in the nest, these learned preferences have been reported to be short-lived or absent as soon as new and equally rewarding alternatives are encountered (Arnold et al. 2021, 2025).

It may be that our bees didn't show a caffeine preference in the assay for various reasons. There were no salient and unique sensory cues provided with each feeder for which caffeine could enhance a conditioned memory, and each feeder offered food of equivalent caloric value. That said, pilot data from our lab shows that pre-exposure to caffeinated food can cause bumblebees to prefer caffeinated food at this concentration over equimolar sucrose under similar exposure conditions and in the absence of visual or olfactory cues, but the feeders were presented at opposite ends of the cage. In this assay, the spatial proximity of the feeders might have meant bees were not sufficiently encouraged to make active feeding decisions. The neurological mechanisms of spatial learning are also quite different from those of taste and smell (Webb & Wystrach 2016) and perhaps therefore less amenable to caffeine's pharmacological influence. In an analogous assay of Drosophila caffeine feeding, where feeders were presented without much spatial separation, preferences were also absent (Kanno et al. 2021). It might be informative to see how subjects behave in the beecam y-maze if distinct visual cues were offered at either arm and/or feeding decisions tracked over longer timescales.

When the preferences of individual subjects were weighted equally, preferences for caffeine were absent. However, when feeding was considered across the population at an absolute level, we found that a very slight but stable preferential feeding ratio was quickly established for the drug which may be reflected in the slightly but consistently steeper gradient of cumulative caffeine duration feeding relative to sucrose. Initial feeding bouts on caffeine tended to be slightly shorter in duration, if anything, suggesting this was not due to the presence of a learned taste cue (in the same way that humans acquire a taste for the bitter flavour of coffee). This begs the question of whether this again evinces disproportionate drug ingestion by a small subset of addiction-prone individuals. More experiments will be required to start to unpick this.

Nor did scopolamine

Bees did not show preferential scopolamine feeding. If anything, they might have biased their feeding away from it slightly, and first bouts were marginally reduced in duration when scopolamine was present in the food. This seems to imply that the drug did not serve as an addictive reinforcer under these exposure conditions.

In the absence of much literature on the effects of chronic mAChR antagonist exposure on insect brains it's hard to rule out that 3 days of scopolamine exposure was insufficient to produce homeostatic changes in the bee brains. It seems plausible that bees might have experienced some mAChR upregulation but harder to speculate as to the changes in ACh release patterns in synapses to compensate for the neurotransmitter accumulation and reduced neuromodulatory efficacy. Despite their excitatory activity, given that mAChRs are present in much smaller numbers than nAChRs in the insect nervous system (Trimmer 1995) and serve slower, neuromodulatory functions, perhaps we would have expected the extent and consequences of acquired drug tol-

erance to be less pronounced than that of nicotine, whatever the underlying mechanisms. The complete absence of bingeing behaviour, however, seems to suggest that the ingestion of muscarinic antagonists doesn't seem to be a self-reinforcing behaviour. That is not necessarily to say that they are incapable of indirectly modulating reward signalling, and it is interesting to consider why the initial scopolamine aversion was then attenuated throughout the rest of the assay period such that the population-level PI had returned to 0 over the course of the experiment. At a glance this perhaps reveals reversal of a learned taste aversion acquired during pre-exposure. However, that presupposes that bees can taste scopolamine at this concentration. It may also be unlikely as scopolamine blocks conditioned taste aversion learning in mammals (Morin et al. 2021).

Detzel & Wink (1993) also reported that bees in a feeding assay find nectar-relevant scopolamine concentrations deterrent, but our findings seem to clash with those of Sculfort et al. (2021) who reported that the first feeding bouts of drug-naive bees in a two minute assay are longer when scopolamine is present in food at much higher concentrations than ours (∼30mM), and concluded that bees find the drug 'phagostimulatory', or perhaps post-ingestively reinforcing. Both studies do concur that bees are able to detect and respond to scopolamine in food while feeding within a short window of time, and it's intriguing to consider what underlies this discrepancy in response. Our bees were not drug-naive, but as discussed it seems unlikely that they were expressing a learned avoidance behaviour. Perhaps the lack of preference was linked in some other way to the pre-exposure treatment. It also seems possible that a concentration-dependent effect could be at play, though if this were responsible it would be surprising as hormetic drug responses tend to be more beneficial at lower concentrations. Though its nectar presence has not been screened with much taxonomic breadth, scopolamine is found in the nectar of various *Datura*, *Lycium*, *Brumansia* and *Hyoscyamus* species at concentrations ranging from 10uM to 1.3mM (Boros et al. 2010, Kerchner et al. 2015), so there is scope for assaying a wider range of drug concentrations. Ideally this would be done over longer timescales with both drug naive and pre-exposure/ withdrawal treatments before coming to any conclusions about whether scopolamine acts as an addictive agent in these nectars.

Conclusions

The evidence we present here addresses the ecologically interesting and important question of whether plants can make addicts of their pollinators. Our data confirm that bees show initial attraction to field-relevant concentrations of nicotine in nectar, and possibly certain other nAChR agonists, including arecoline; and are the first to identify behaviourally that, consistent with this hypothesis, prolonged exposure and withdrawal to these nectars increases the rate of drug intake. However, pre-exposure to and withdrawal from thiamethoxam, scopolamine, and caffeine at presumed field-relevant concentrations did not produce evidence of addiction in this context. Further research will be required to understand how addiction to nectar laced with cholinergic drugs of various mechanisms of action and at various concentrations could arise under natural conditions, ideally through voluntary exposure and in the presence of more field-relevant learning cues.

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Data availability

The analysis script and blinds .csv files are available at https://github.com/annadorling in the Chapter 4 repo. The dataframes for experiments 4a and 4b are accessible from Google Drive.

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SUPPLEMENTARY INFORMATION

Experiment 4a (nicotine)

Table S1 | ANOVA test of fixed effect parameter significance in mixed models of cumulative feeding behaviour in the no choice group, experiment 4a; where 'S1' and 'S2' are randomly assigned sucrose feeders

`lmer(var ~ Solution + (1|bee_ID)`

(a) Total duration fed	NumDF	DenDF	*F*	*p*
Solution	1	48	0.200	0.657
(b) Total feeding attempts	NumDF	DenDF	*F*	*p*
Solution	1	26.0	0.348	0.560
(c) Feeding rate (T50) by duration	NumDF	DenDF	*F*	*p*
Solution	1	48	0.534	0.468
(d) Feeding rate (T50) by feeds	NumDF	DenDF	*F*	*p*
Solution	1	24.4	0.406	0.530

Table S2 | ANOVA test of fixed effect parameter significance in mixed models of cumulative feeding behaviour in the choice groups, experiment 4a; where Treatment = 'Pre-exposed' or 'Naive', and Solution = 'nicotine' or 'sucrose'

`lmer(var ~ Solution * treatment + (1|bee_ID)`

	Full model				**Simplified model**			
(a) Total duration fed	NumDF	DenDF	F	p	NumDF	DenDF	F	p
Solution	1	33.7	1.75	0.195	1	34.9	1.95	0.171
Treatment	1	34.3	0.011	0.917	1	34.4	0.014	0.908
Solution × Treatment	1	33.7	0.427	0.518				
(b) Total feeding attempts	NumDF	DenDF	F	p	NumDF	DenDF	F	p
Solution	1	34.0	0.252	0.619	1	35.1	0.378	0.543
Treatment	1	35.7	0.637	0.430	1	35.8	0.613	0.439
Solution × Treatment	1	34.0	1.61	0.213				
(c) Rate (T50) by duration	NumDF	DenDF	F	p	NumDF	DenDF	F	p
Solution	1	69	20.8	< 0.0001 ***	1	70	21.3	< 0.0001 ***
Treatment	1	69	0.133	0.717	1	70	0.135	0.715
Solution × Treatment	1	69	0.001	0.980				
(d) Rate (T50) by feeds	NumDF	DenDF	F	p	NumDF	DenDF	F	p
Solution	1	69	9.93	0.002 **	1	70	10.4	0.002 **
Treatment	1	69	0.297	0.588	1	70	0.294	0.589
Solution × Treatment	1	69	0.120	0.730				

Time-binned rate data

Table S3a | Parameter significance for binned no choice duration

```
mod_ctrl_duration <- glmmTMB(seconds_fed∼Solution * as.factor(time_bin) + (1|bee_ID),
family = tweedie())
```

	Full model			Simplified model		
Parameter	χ^2	df	p	χ^2	df	p
Solution	0.064	1	0.800	0.064	1	0.800
Time bin	19.2	5	0.002 **	19.0	5	0.002 **
Solution × time bin	3.55	5	0.616			

Table S3b | Pairwise comparisons of the duration for which individuals in the no choice treatment consumed each solution at each time bin; where 'S1' and 'S2' are randomly assigned sucrose feeders

```
emm <- emmeans(mod_ctrl_duration∼Solution|time_bin)
contrast(emm, method = "pairwise")
```

Time bin	Contrast	Estimate	SE	df	z-ratio	p-value
0-4h	S1–S2	-0.251	0.343	Inf	-0.732	0.464
4-8h	S1–S2	0.377	0.393	Inf	0.960	0.337
8-12h	S1–S2	0.171	0.398	Inf	0.429	0.668
12-16h	S1–S2	-0.566	0.436	Inf	-1.30	0.194
16-20h	S1–S2	0.181	0.458	Inf	0.395	0.693
20-24h	S1–S2	-0.142	0.401	Inf	-0.354	0.723

Table S3c | Parameter significance for binned no choice feeds

```
mod_ctrl_feeds <- glmmTMB(feeds∼Solution * as.factor(time_bin) + (1|bee_ID), family = tweedie())
```

	Full model			Simplified model		
Parameter	χ^2	df	p	χ^2	df	p
Solution	0.065	1	0.799	0.057	1	0.811
Time bin	40.7	5	$<$ 0.001 ***	40.6	5	$<$ 0.001 ***
Solution × time bin	8.95	5	0.111			

Table S3d | Pairwise comparisons of the number of feeds individuals in the no choice treatment made on each solution at each time bin; where 'S1' and 'S2' are randomly assigned sucrose feeders

```
mod <- glmmTMB(seconds_fed∼Solution * as.factor(time_bin) + (1|bee_ID), family = tweedie())
emm <- emmeans(mod∼Solution|time_bin)
contrast(emm, method = "pairwise")
```

Time bin	Contrast	Estimate	SE	df	z-ratio	p-value
0-4h	S1–S2	-0.024	0.267	Inf	-0.090	0.928
4-8h	S1–S2	0.578	0.344	Inf	1.68	0.092 .
8-12h	S1–S2	-0.155	0.323	Inf	-0.481	0.630
12-16h	S1–S2	-0.843	0.392	Inf	-2.15	0.031 *
16-20h	S1–S2	0.351	0.436	Inf	0.805	0.421
20-24h	S1–S2	0.266	0.334	Inf	0.796	0.426

Table S4a | Parameter significance for binned pre-exposed duration

```
mod <- glmmTMB(seconds_fed∼Solution * as.factor(time_bin) + (1|bee_ID), family = tweedie())
```

	Full model			Simplified model		
Parameter	χ^2	df	p	χ^2	df	p
Solution	0.849	1	0.357	0.807	1	0.369
Time bin	25.2	5	< 0.001 ***	24.4	5	< 0.001 ***
Solution $\times$ time bin	8.80	5	0.117			

Table S4b | Pairwise comparisons of the duration for which individuals in the pre-exposed treatment consumed either solution at each time bin, where 'N' = 1 M sucrose + 25 µM nicotine and 'S' = 1 M sucrose

```
mod <- glmmTMB(seconds_fed∼Solution * as.factor(time_bin) + (1|bee_ID), family = tweedie())
emm <- emmeans(mod∼Solution|time_bin)
contrast(emm, method = "pairwise")
```

Time bin	Contrast	Estimate	SE	df	z-ratio	p-value
0-4h	N–S	0.957	0.390	Inf	2.45	0.014 *
4-8h	N–S	0.664	0.479	Inf	1.39	0.166
8-12h	N–S	-0.188	0.474	Inf	-0.397	0.692
12-16h	N–S	-0.258	0.492	Inf	-0.526	0.599
16-20h	N–S	-0.200	0.519	Inf	-0.385	0.701
20-24h	N–S	-0.559	0.529	Inf	-1.06	0.291

Table S4c | Parameter significance for binned pre-exposed feeds

```
mod <- glmmTMB(feeds∼Solution * as.factor(time_bin) + (1|bee_ID), family = tweedie())
```

	Full model			Simplified model		
Parameter	χ^2	df	p	χ^2	df	p
Solution	0.556	1	0.456	0.558	1	0.455
Time bin	35.4	5	< 0.001 ***	34.5	5	< 0.001 ***
Solution $\times$ time bin	7.02	5	0.219			

Table S4d | Pairwise comparisons of the number of feeds individuals in the pre-exposed treatment made on each solution at each time bin, where 'N' = 1 M sucrose + 25 µM nicotine and 'S' = 1 M sucrose

```
mod <- glmmTMB(feeds∼Solution * as.factor(time_bin) + (1|bee_ID), family = tweedie())
emm <- emmeans(mod∼Solution|time_bin)
contrast(emm, method = "pairwise")
```

Time bin	Contrast	Estimate	SE	df	z-ratio	p-value
0-4h	N–S	0.745	0.309	Inf	2.41	0.016 *
4-8h	N–S	0.241	0.400	Inf	0.602	0.547
8-12h	N–S	-0.222	0.403	Inf	-0.551	0.582
12-16h	N–S	-0.101	0.424	Inf	-0.239	0.811
16-20h	N–S	-0.145	0.429	Inf	-0.337	0.736
20-24h	N–S	-0.464	0.476	Inf	-0.975	0.330

Table S5a | Parameter significance for binned naive duration

```
mod <- glmmTMB(seconds_fed∼Solution * as.factor(time_bin) + (1|bee_ID), family = tweedie())
```

	Full model			Simplified model		
Parameter	χ^2	df	p	χ^2	df	p
Solution	0.043	1	0.836	0.037	1	0.849
Time bin	22.3	5	< 0.001 ***	22.0	5	< 0.001 ***
Solution $\times$ time bin	6.70	5	0.244			

Table S5b | Pairwise comparisons of the duration for which individuals in the naive treatment consumed each solution at each time bin, where 'N' = 1 M sucrose + 25 µM nicotine and 'S' = 1 M sucrose

```
mod <- glmmTMB(seconds_fed∼Solution * as.factor(time_bin) + (1|bee_ID), family = tweedie())
emm <- emmeans(mod∼Solution|time_bin)
contrast(emm, method = "pairwise")
```

Time bin	Contrast	Estimate	SE	df	z-ratio	p-value
0-4h	N–S	0.659	0.391	Inf	1.69	0.092 .
4-8h	N–S	0.275	0.446	Inf	0.616	0.538
8-12h	N–S	-0.457	0.525	Inf	-0.870	0.384
12-16h	N–S	-0.387	0.460	Inf	-0.841	0.400
16-20h	N–S	0.258	0.489	Inf	0.528	0.597
20-24h	N–S	-0.811	0.595	Inf	-1.36	0.173

Table S5c | Parameter significance for binned naive feeds

```
mod <- glmmTMB(feeds∼Solution * as.factor(time_bin) + (1|bee_ID), family = tweedie())
```

	Full model			Simplified model		
Parameter	χ^2	df	p	χ^2	df	p
Solution	0.945	1	0.331	1.01	1	0.315
Time bin	40.1	5	< 0.001 ***	40.1	5	< 0.001 ***
Solution $\times$ time bin	5.62	5	0.346			

Table S5d | Pairwise comparisons of the number of feeds individuals in the naive treatment made on each solution at each time bin, where 'N' = 1 M sucrose + 25 µM nicotine and 'S' = 1 M sucrose

```
mod <- glmmTMB(seconds_fed∼Solution * as.factor(time_bin) + (1|bee_ID), family = tweedie())
emm <- emmeans(mod∼Solution|time_bin)
contrast(emm, method = "pairwise")
```

Time bin	Contrast	Estimate	SE	df	z-ratio	p-value
0-4h	N–S	0.167	0.286	Inf	0.583	0.560
4-8h	N–S	0.126	0.365	Inf	0.345	0.730
8-12h	N–S	-0.870	0.469	Inf	-1.86	0.064 .
12-16h	N–S	-0.309	0.431	Inf	-0.717	0.473
16-20h	N–S	-0.082	0.388	Inf	-0.210	0.833
20-24h	N–S	-0.688	0.474	Inf	-1.45	0.146

First bout duration model output and parameter significance

Table S6a | Linear model output of the difference in first bout duration between the solutions, by group

`summary(lm(dif_firstbout~Treatment))`

Coefficient	Estimate	SE	*t*	*p*
`Intercept`	-3.67	8.69	-0.422	0.674
`TreatmentPre-exposed`	7.16	12.9	0.554	0.581
`TreatmentNaive`	-3.74	13.6	-0.276	0.784

Table S6b | Parameter significance for linear model of the difference in first bout duration between the solutions

`anova(lm(dif_firstbout~Treatment))`

Parameter	df	F	*p*
`Treatment`	2	0.316	0.730
`Residuals`	55		

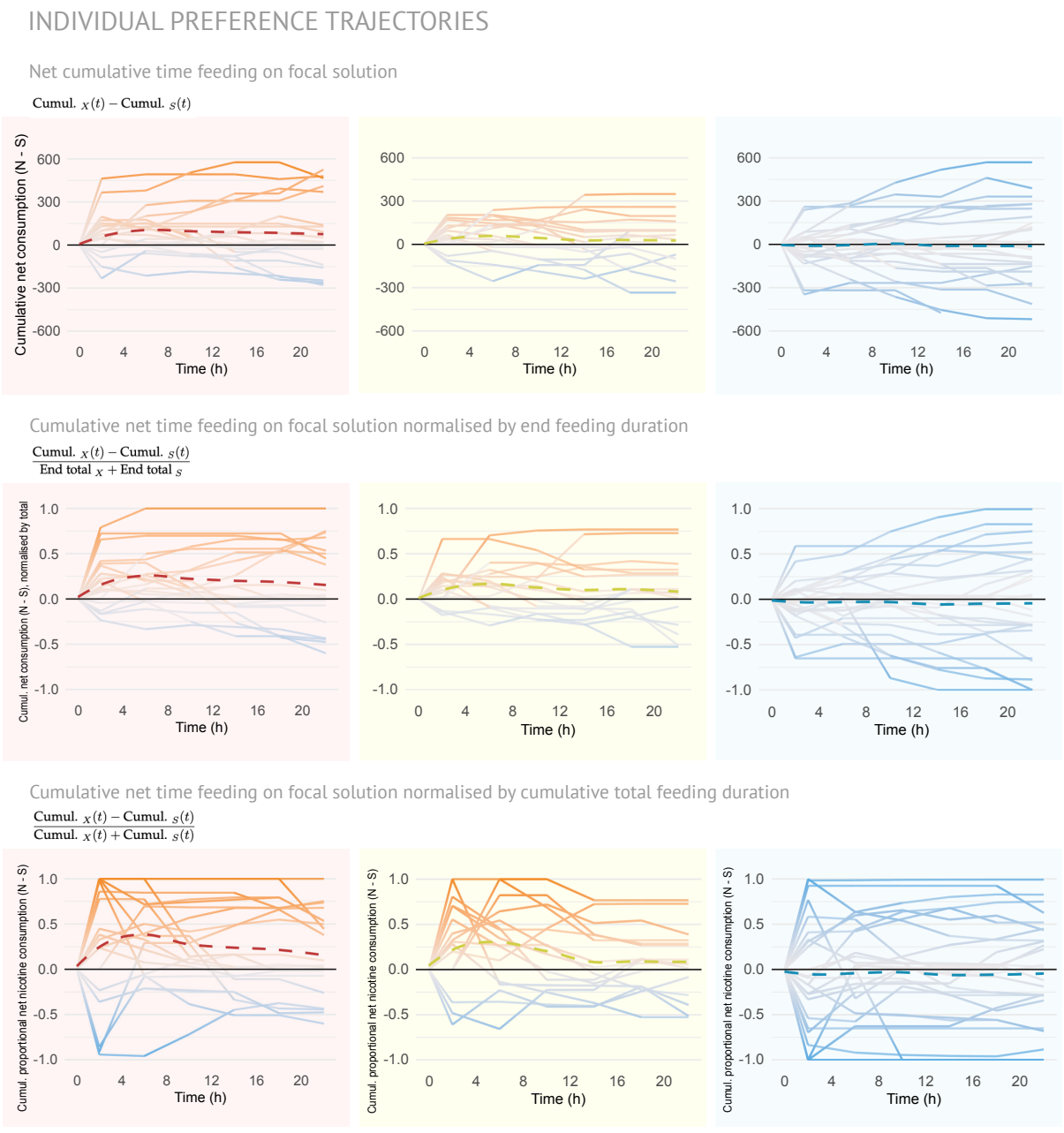


Figure S7 | **Alternative individual preference index trajectories**. All were calculated across 4h time bins using cumulative feeding duration data on either solution. The formulae on top of each row denotes how each were calculated. In the case of columns one and two, the focal solution was 1M sucrose + 25µM nicotine; and in the third was 1M sucrose, randomly assigned as solution 'S1'. Data were filtered to exclude 4 bees that consumed food in unusually small quantities using a 10 second threshold. n = 19 (pre-exposed), 16 (naive), 26 (no choice).

Experiment 4b (other drugs)

Cumulative feeding

Table S8 | ANOVA test of fixed effect parameter significance in mixed models of cumulative feeding behaviour in experiment 4b

a. `lmer(sqrt(24h_duration) ~ Solution * Treatment + (1 | Bee_ID), data = total_durations)`
b. `lmer(sqrt(24h_feeds) ~ Solution * Treatment + (1 | Bee_ID), data = total_feeds)`
c. `lmer(midpoint ~ Solution * Treatment + (1 | Bee_ID), data = duration_midpoints)`
d. `lmer(midpoint ~ Solution * Treatment + (1 | Bee_ID), data = feed_count_midpoints)`

(a) Total duration fed	NumDF	DenDF	F	p
Solution_type	1	221	0.091	0.764
Treatment	3	221	0.738	0.530
Solution_type × Treatment	3	221	1.08	0.359
(b) Total feeding attempts	NumDF	DenDF	F	p
Solution_type	1	113	0.003	0.955
Treatment	3	116	1.46	0.228
Solution_type × Treatment	3	113	1.65	0.183
(c) Rate (T50) by duration	NumDF	DenDF	F	p
Solution_type	1	221	0.659	0.418
Treatment	3	221	0.605	0.612
Solution_type × Treatment	3	221	2.05	0.108
(d) Rate (T50) by feeds	NumDF	DenDF	F	p
Solution_type	1	221	0.214	0.644
Treatment	3	221	1.77	0.155
Solution_type × Treatment	3	221	2.01	0.113

Time-binned rate data

Table S9a | Parameter significance for binned arecoline duration

`arecoline_duration_mod <- glmmTMB(seconds_fed~Solution * as.factor(time_bin) + (1|bee_ID), family = tweedie())`

	Full model			**Simplified model**		
Parameter	χ^2	df	p	χ^2	df	p
Solution	2.09	1	0.148	2.07	1	0.151
Time bin	14.4	5	0.013 *	14.3	5	0.014 *
Solution × time bin	4.91	5	0.427			

Table S9b | Pairwise comparisons of the duration for which individuals in the arecoline treatment consumed each solution at each time bin, where 'A' = 1 M sucrose + 10 µM arecoline and 'S' = 1 M sucrose

```
emm <- emmeans(arecoline_duration_mod ~ Solution | time_bin)
contrast(emm, method = "pairwise")
```

Time bin	Contrast	Estimate	SE	df	z-ratio	p-value
0-4h	A–S	0.615	0.377	Inf	1.63	0.103
4-8h	A–S	0.246	0.411	Inf	0.598	0.550
8-12h	A–S	0.187	0.425	Inf	0.439	0.660
12-16h	A–S	-0.175	0.476	Inf	-0.367	0.714
16-20h	A–S	-0.290	0.435	Inf	-0.666	0.505
20-24h	A–S	0.847	0.474	Inf	1.79	0.074 .

Table S9c | Parameter significance for binned arecoline feeds

```
arecoline_feeds_mod <- glmmTMB(number_feeds ~
Solution * as.factor(time_bin) + (1|bee_ID),
family = tweedie())
```

	Full model			**Simplified model**		
Parameter	χ^2	df	p	χ^2	df	p
Solution	4.80	1	0.028 *	4.72	1	0.030 *
Time bin	48.4	5	$<$ 0.001 ***	48.0	5	$<$ 0.001 ***
Solution $\times$ time bin	6.19	5	0.288			

Table S9d | Pairwise comparisons of the number of feeds individuals in the arecoline treatment made from each solution at each time bin, where 'A' = 1 M sucrose + 10 µM arecoline and 'S' = 1 M sucrose

```
emm <- emmeans(arecoline_feeds_mod, ~ Solution | time_bin)
contrast(emm, method = "pairwise")
```

Time bin	Contrast	Estimate	SE	df	z-ratio	p-value
0-4h	A–S	0.594	0.275	Inf	2.16	0.031 *
4-8h	A–S	0.480	0.355	Inf	1.35	0.177
8-12h	A–S	0.247	0.364	Inf	0.677	0.498
12-16h	A–S	0.318	0.407	Inf	0.780	0.435
16-20h	A–S	-0.553	0.403	Inf	-1.37	0.170
20-24h	A–S	0.534	0.427	Inf	1.25	0.211

Table S10a | Parameter significance for binned scopolamine duration

```
scopolamine_duration_mod <- glmmTMB(seconds_fed ~
 Solution * as.factor(time_bin) + (1|bee_ID),
family = tweedie())
```

	Full model			**Simplified model**		
Parameter	χ^2	df	p	χ^2	df	p
Solution	0.028	1	0.868	0.028	1	0.867
Time bin	20.5	5	0.001 **	20.4	5	0.001 **
Solution $\times$ time bin	1.78	5	0.879			

Table S10b | Pairwise comparisons of the duration for which individuals in the scopolamine treatment consumed each solution at each time bin

```
emm <- emmeans(scopolamine_duration_mod ~ Solution | time_bin)
contrast(emm, method = "pairwise")
```

Time bin	Contrast	Estimate	SE	df	z-ratio	p-value
0-4h	Scop–Suc	-0.255	0.320	Inf	-0.796	0.426
4-8h	Scop–Suc	0.220	0.350	Inf	0.628	0.530
8-12h	Scop–Suc	0.105	0.377	Inf	0.279	0.781
12-16h	Scop–Suc	-0.299	0.404	Inf	-0.739	0.460
16-20h	Scop–Suc	0.146	0.392	Inf	0.372	0.710
20-24h	Scop–Suc	-0.041	0.366	Inf	-0.113	0.910

Table S10c | Parameter significance for binned scopolamine feeds

```
scopolamine_feeds_mod <- glmmTMB(number_feeds~Solution * as.factor(time_bin) + (1|bee_ID),
family = tweedie())
```

	Full model			**Simplified model**		
Parameter	χ^2	df	p	χ^2	df	p
Solution	0.347	1	0.560	0.348	1	0.555
Time bin	37.5	5	< 0.001 ***	37.4	5	< 0.001 ***
Solution $\times$ time bin	2.28	5	0.810			

Table S10d | Pairwise comparisons of the number of feeds individuals in the scopolamine treatment made from each solution at each time bin

```
emm <- emmeans(scopolamine_feeds_mod, ~ Solution | time_bin)
contrast(emm, method = "pairwise")
```

Time bin	Contrast	Estimate	SE	df	z-ratio	p-value
0-4h	Scop–Suc	-0.253	0.243	Inf	-1.04	0.298
4-8h	Scop–Suc	0.147	0.303	Inf	0.485	0.628
8-12h	Scop–Suc	-0.039	0.310	Inf	-0.126	0.900
12-16h	Scop–Suc	-0.383	0.353	Inf	-1.08	0.279
16-20h	Scop–Suc	0.024	0.318	Inf	0.076	0.940
20-24h	Scop–Suc	0.103	0.310	Inf	0.331	0.741

Table S11a | Parameter significance for binned TMX duration

```
TMX_duration_mod <- glmmTMB(seconds_fed~Solution * as.factor(time_bin) + (1|bee_ID),
family = tweedie())
```

	Full model			**Simplified model**		
Parameter	χ^2	df	p	χ^2	df	p
Solution	1.17	1	0.280	1.15	1	0.284
Time bin	20.3	5	0.001 **	20.0	5	0.001 **
Solution $\times$ time bin	6.04	5	0.303			

Table S11b | Pairwise comparisons of the duration for which individuals in the TMX treatment consumed each solution at each time bin

```
emm <- emmeans(TMX_duration_mod, ~ Solution|time_bin)
contrast(emm, method = "pairwise")
```

Time bin	Contrast	Estimate	SE	df	z-ratio	p-value
0-4h	TMX–Suc	-0.527	0.334	Inf	-1.58	0.114
4-8h	TMX–Suc	0.425	0.356	Inf	1.20	0.232
8-12h	TMX–Suc	-0.376	0.407	Inf	-0.923	0.356
12-16h	TMX–Suc	-0.432	0.414	Inf	-1.04	0.297
16-20h	TMX–Suc	0.224	0.375	Inf	0.596	0.551
20-24h	TMX–Suc	-0.403	0.410	Inf	-0.983	0.326

Table S11c | Parameter significance for binned TMX feeds

```
TMX_feeds_mod <- glmmTMB(seconds_fed ~ Solution * as.factor(time_bin) + (1|bee_ID),
family = tweedie())
```

	Full model			**Simplified model**		
Parameter	χ^2	df	p	χ^2	df	p
Solution	3.00	1	0.083 .	3.00	1	0.083 .
Time bin	38.8	5	$<$ 0.001 ***	38.4	5	$<$ 0.001 ***
Solution $\times$ time bin	4.31	5	0.506			

Table S11d | Pairwise comparisons of the number of feeds individuals in the TMX treatment made from each solution at each time bin

```
emm <- emmeans(TMX_feeds_mod ~ Solution|time_bin)
contrast(emm, method = "pairwise")
```

Time bin	Contrast	Estimate	SE	df	z-ratio	p-value
0-4h	TMX–Suc	-0.423	0.245	Inf	-1.73	0.084 .
4-8h	TMX–Suc	0.199	0.292	Inf	0.680	0.497
8-12h	TMX–Suc	-0.205	0.322	Inf	-0.636	0.525
12-16h	TMX–Suc	-0.326	0.346	Inf	-0.943	0.346
16-20h	TMX–Suc	0.022	0.331	Inf	0.067	0.946
20-24h	TMX–Suc	-0.545	0.339	Inf	-1.61	0.108

Table S12a | Parameter significance for binned caffeine duration

```
caffeine_duration_mod <- glmmTMB(seconds_fed~Solution * as.factor(time_bin) + (1|bee_ID),
family = tweedie())
```

	Full model			**Simplified model**		
Parameter	χ^2	df	p	χ^2	df	p
Solution	0.427	1	0.513	0.427	1	0.514
Time bin	16.9	5	0.005 **	16.8	5	0.005 **
Solution $\times$ time bin	0.838	5	0.975			

Table S12b | Pairwise comparisons of the duration for which individuals in the caffeine treatment consumed each solution at each time bin, where 'C' = 1 M sucrose + 100 µM caffeine and 'S' = 1 M sucrose

```
emm <- emmeans(caffeine_duration_mod, ~ Solution|time_bin)
contrast(emm, method = "pairwise")
```

Time bin	Contrast	Estimate	SE	df	z-ratio	p-value
0-4h	C–S	-0.024	0.337	Inf	-0.072	0.943
4-8h	C–S	0.026	0.374	Inf	0.068	0.946
8-12h	C–S	0.062	0.361	Inf	0.171	0.864
12-16h	C–S	0.145	0.405	Inf	0.358	0.721
16-20h	C–S	0.076	0.394	Inf	0.192	0.848
20-24h	C–S	0.434	0.418	Inf	1.04	0.300

Table S12c | Parameter significance for binned caffeine feeds

```
caffeine_feeds_mod <- glmmTMB(total_feeds ~
Solution * as.factor(time_bin) + (1|bee_ID),
family = tweedie())
```

	Full model			**Simplified model**		
Parameter	χ^2	df	p	χ^2	df	p
Solution	0.024	1	0.878	0.030	1	0.862
Time bin	29.8	5	< 0.001 ***	29.9	5	< 0.001 ***
Solution $\times$ time bin	5.27	5	0.384			

Table S12d | Pairwise comparisons of the number of feeds individuals in the caffeine treatment made from each solution at each time bin, where 'C' = 1 M sucrose + 100 µM caffeine and 'S' = 1 M sucrose

```
emm <- emmeans(caffeine_feeds_mod~Solution|midpoint)
contrast(emm, method = "pairwise")
```

Time bin	Contrast	Estimate	SE	df	z-ratio	p-value
0-4h	C–S	-0.035	0.247	Inf	-0.142	0.887
4-8h	C–S	-0.036	0.312	Inf	-0.114	0.909
8-12h	C–S	-0.062	0.288	Inf	-0.214	0.831
12-16h	C–S	-0.300	0.340	Inf	-0.881	0.378
16-20h	C–S	-0.061	0.312	Inf	-0.195	0.845
20-24h	C–S	0.714	0.341	Inf	2.10	0.036 *

First bout duration model output and parameter significance

Table S13a | Linear model output of the difference in first bout duration between the solutions, by group

```
summary(lm(dif_firstbout ~ Treatment))
```

Coefficient	Estimate	SE	*t*	*p*
`Intercept`	6.14	6.54	0.939	0.350
`TreatmentScopolamine`	-17.0	8.79	-1.93	0.056 .
`TreatmentTMX`	-5.20	9.08	-0.573	0.568
`TreatmentCaffeine`	-9.64	9.25	-1.04	0.300

Table S13b | Parameter significance for linear model of the difference in first bout duration between the solutions

`anova(lm(dif_firstbout~Treatment))`

Parameter	df	F	*p*
`Treatment`	3	1.36	0.260
`Residuals`	104		

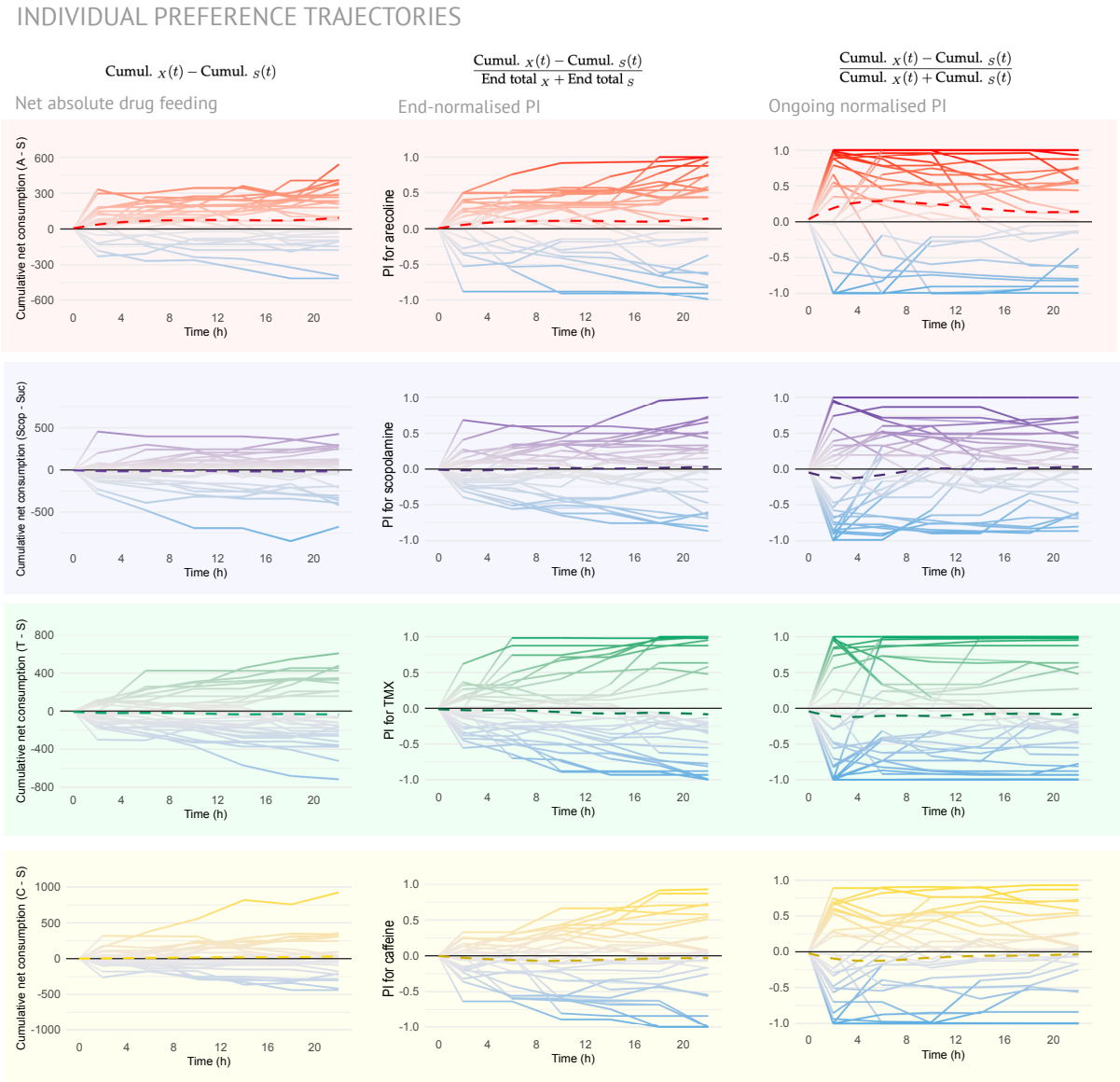


Figure S14 | **Extended individual preference trajectories**. These each show how the direction of feeding choices made by each individual evolved over time according to a different formulae. These are displayed above each column and used data about the cumulative time spent feeding in seconds on either solution across a series of 4h time bins. The focal solution, X, in each case was 1M sucrose + either 10µM arecoline (top row), 80µM scopolamine (second row), 35nM TMX (third row) or 100µM caffeine (bottom row). Data were filtered to exclude 3 bees that consumed food in unusually small quantities. n = 28 (arecoline), 30 (scopolamine), 32 (TMX), 28 (caffeine).

Chapter 5 | General Discussion

When I started my DPhil, the idea that bees might become addicted to neuroactive nectar felt like low-hanging fruit. I now see that it's difficult to test rigorously, not least because doing so demands a broad experimental toolkit and knowledge base. It's a question that sits at a busy juncture of neurophysiology, phytochemistry, pollination ecology, cognition, and behaviour; and the study of addiction in insect models is still in its infancy.

My aim with this project was ultimately to identify whether bees can become addicted to nectar drugs that influence cholinergic signalling. Broadly, the approach taken throughout this work is two-pronged. Drugs of abuse are addictive – that is, they have the potential to produce addiction – because they synthetically engage the reward system. On one hand, I therefore look to see if nicotine has addictive potential in bees by studying its effects on learning, memory, reward and preference. Concurrently, I have investigated whether pre-exposing bees to nicotine and subsequently depriving them of it for 24 h yields behavioural evidence that they had become addicted.

In the following sections I synthesise the findings emerging from each of these lines of inquiry and suggest some future directions.

On the addictive potential of nicotine: cognition, reward, and preference

Recent work has indicated that nicotine in nectar can alter normative processes of reward valuation in bees because it encodes preferences for drug-laced food over a non-drugged but otherwise identical alternative (Singaravelan et al. 2005) and enhances the speed of a learning task in free flight (Baracchi et al. 2017). However, these nicotine biases haven't always been repeatable: Tiedeken et al. (2014) found that the effect was absent in a two-way choice assay over 24 h, and people in our lab performed an unpublished repeat of the work by Baracchi et al. (2017), finding no effect.

Some of my results mirror this ambiguity. In the choice feeding assay in chapter 2 (experiment 2a), nicotine preferences were absent in the naive bees but the drug appeared to influence their responses in subtle and often marginal ways during the olfactory conditioning assay (experiments 2bi and 2bii). Feeding preferences in the pre-exposed bees were slight and only significant after a period of withdrawal. Similarly, in the first experiment presented in chapter 4 (experiment 4a), nicotine preference measured at the population level by averaging across individual preference indices was insignificant by both mass and duration, regardless of pre-exposure.

Beginning this project, I somewhat naively expected that with the PER and choice assays at my disposal I'd have all the necessary means to determine whether nicotine-laced nectar could be addictive to bees and then I could quickly move on to surveying the nectar distributions of other drugs and their addictive properties. Eventually the idea occurred to me that the equivocal findings from these experiments could result from treating groups of bees as individual replicates (as in the choice assays) and/or otherwise averaging responses across the population, which could obscure meaningful differences between individuals and through time.

When preference was measured in the novel way in chapter 4, i.e. continuously, treating subjects as the unit of replication, a nicotine bias was detected in the first few hours which was attenuated through time but remained slightly present, even at 24 h. This was absent in the data that had been expressed as an index between -1 and 1. This suggests that on an absolute scale, rather than when individuals are weighted equally, there was more nicotine feeding. The preference trajectories from individual bees bear this out, indicating that a subset of individuals may be amenable to developing strong nicotine preferences. This could also account for some of the contradictory results across and within (Baracchi et al. 2017, Sokolowski et al. 2012) bee drug preference studies.

What is a feeding preference? Focussing foraging effort on food of higher quality might be more relevant to bees in costly or competitive contexts. Under conditions of food abundance, however, such as in these preference assays – and perhaps also those conducted in free flight using colonies in a well-fed state and with relatively large reward volumes (Baracchi et al. 2017) – a survival pressure to make correct choices is absent. Additionally, the data in chapter 3 show that first bouts are much longer in duration than the average bout and bees seem to enter the assays in a state of hunger. Under these conditions (at least initially), perhaps choices are therefore mostly made on the

grounds of a mix of chance and immediate hedonic feedback as bees fill up on whichever option stochastically presents itself first. For this reason, I suspect that the identified nicotine preferences lend greater support to an enhanced hedonic quality of nicotine-laced food than a pharmacological influence on the Kenyon cells, though this would need further exploration. Whatever its cause, the initial nicotine bias is somewhat transient and the magnitude of the measured 'preference' falls over time. The strength of the preference readout will therefore correspond to the duration of the choice phase, and I suggest this may also explain some of the disagreement in the literature.

The PER work in chapter 2 was intended to determine the drug's influence on the processes of learning and memory. We see a possible signature of drug-enhanced performance in the long term recall data in round 1 and the learning data in round 2. However, it was difficult to conclude much with confidence from this data given its collection across different years and the difficulties we had in standardising the motivational states of bees. Unlike in the untrammelled nicotine self-administration scenario, reward delivery was tightly constrained in its volume (0.4 µL at each presentation) and timing. This exposure pattern was therefore very different, and potentially more field-relevant. Given the much lower drug dose it also seems likely that there will have been less of an impact on the mechanisms of food reward learning. Determining how nicotine might alter the dynamics of floral learning, recall, and choice in the field specifically will still be important, because *Nicotiana* nectar volumes per flower vary across orders of magnitude (∼0.1-10 µL, according to Tiedge & Lohaus (2017)). A careful, concurrently collected repeat of this PER assay would nonetheless help to reveal the relative contributions of nicotine to reinforcement, learning, and memory. Drug differences may be more detectable if the task were made slightly harder – perhaps by reducing the number of trials, sugar concentration of the reward, or the intertrial interval.

Did bees become addicted with pre-exposure?

Addictive drugs share an ability to hijack and dysregulate the brain's reward and learning systems to create strong reinforcement loops which result in recurrent drug use. Addiction itself is quite loosely defined but is generally agreed to result from repeated drug exposure and involve a shift from volitional to compulsive (i.e. escalating and costly) drug use. Physiological dependence develops as a side effect of repeated exposure. This compounds drug-seeking motivation, but compulsive drug use is attributed to the developent of a super-rewarding, highly persistent memory rather than the desire to maintain homeostasis (Hyman et al. 2006) and it is this which is thought to explain relapse behaviour in addicts (DiFranza 2015, Hyman 2005). Withdrawal is nonetheless recognised as a diagnostic feature of the addictive state and is therefore also important to study, even if just to corroborate accounts of compulsive drug-seeking.

Built into all of these experiments were tests of how forced pre-exposure to and withdrawal from nicotine would change the behavioural responses of bees in comparison to those that were naive to it. In my experiments, I was looking for evidence that these bees showed (i) more extensive drug seeking than naive or non-withdrawn bees, or (ii) impaired performance in the PER assay when deprived of the drug during conditioning, which could be symptomatic of withdrawal-induced impairments to their cognition or decreased natural reward sensitivity due to dysregulation of their reward systems.

Chapter 2 finds evidence of both withdrawal and escalating drug use in honeybees following nicotine pre-exposure. In the preference assay, previous exposure to nicotine biased honeybee feeding decisions in favour of the drug and this resulted in a significant preference after 24 h of withdrawal. The accompanying PER results suggest that this may have been caused by changes to the processes of reward valuation rather than pre-exposed bees simply requiring a higher dose to obtain the same hedonic effect. When in a state of withdrawal, pre-exposed bees showed analogous differences in the ways they responded to either conditioned reward type: those trained on sucrose consistently performed the worst, whereas those receiving nicotine outperformed the other groups. Nicotine-laced reward thus didn't just rescue performance under withdrawal – it seemed to boost performance in a way that implies it was more highly valued by bees in this state. Whether the poor performance of bees in the drug-deprived group was due either to devalution of natural reward or withdrawal-induced impairments to their cognition (or a combination of the two) is unclear. Trends in the survival data from this assay indicate that it's likely that bees were experiencing some degree of stress under the withdrawal treatment, so the latter explanation certainly seems plausible.

The results in chapter 4 validate, in bumblebees, that drug self-administration is increased with pre-exposure and withdrawal; but the initial nicotine-seeking behaviour was expressed

by naive bees too. It is difficult to conclude with confidence that this more extensive self-administration in the pre-exposed group was a compulsive addictive symptom because bees did not have to bear costs of accessing the drug. It seems likely that they were withdrawn given the previous results, but a similar upward translation of the nicotine feeding curve could have resulted if bees had developed tolerance but not addiction to the drug and thus were in need of higher doses to achieve the same hedonic effect. The individual trajectories indicated that a subset of pre-exposed bumblebees sustained moderate to strong preferences for both nicotine and arecoline throughout the monitoring period, which was less true for bees in the naive group. This perhaps suggests that some bees might have been seeking the drug compulsively, but more experiments will be required to test this.

Engineering crop preferences

Commercially bred bumblebee colonies can be a useful resource to farmers (Muñiz et al. 2025) but ultimately their efficiency often limits crop yield and there is plenty of room for pollination services to be optimised (Martin et al. 2019). In view of work from our lab showing that caffeine-laced nectar can enhance the acquisition of an olfactory memory and improve its retention into the long term (Wright et al. 2013) there has been recent effort to 'prime' pollinators, or condition crop preferences, by feeding commercially sourced bumblebee colonies with caffeinated food in the nest while exposing them to the target odour (Arnold et al. 2021, 2025, de Ibarra & Rands 2021).

There are some signs that this could work, but the effect is weak and temporary. By way of a practical suggestion (some might query the ethics of this practice of drugging pollinators for profit), exposing bees to nicotine or arecoline may be more effective. As discussed in chapter 4, these drugs produce a transient preference too but the effect is likely to be stronger.

Other future directions

Identifying compulsion

It would be useful to demonstrate that previous exposure induces bees to seek drugs in the face of costs. Baracchi et al. (2017) identified that bumblebees conditioned to associate one colour with nicotine-laced reward and another with water were more likely to remain faithful to the previously drugged flowers in a reversal learning test. A more rigorous assay of drug-seeking motivation would ideally decouple the volitional aspect of decision-making from the potentially confounding processes of learning and memory, perhaps by measuring bees' willingness to shoulder costs in obtaining a particular reward. For instance, bees will preferentially collect nectar that is energetically demanding to access if it is of a sufficiently higher quality, but will forage from flowers with a lower handling time if the difference is low (Pattrick et al. 2023, Giray et al. 2015). By identifying their switch points, it may be possible to demonstrate that pre-exposed bees are more motivated to seek nicotine. Costs of accessing a reward could potentially take many analogous forms, such as electric shocks (Kaun et al. 2011) or bitter tastants.

Understanding withdrawal

The experiments presented in chapter 2 show that 24 h of nicotine withdrawal caused bees to self-administer more nicotine. In order to better understand the conditions that produce physiological addictions to these drugs in bees it might be helpful to determine the time course and behavioural symptoms of nicotine withdrawal. One way might be to pre-expose bees to nicotine for three days and then assay the extent of the nicotine bias in their feeding after withdrawal periods of different durations. In chapter 4, the disparity in the amount of nicotine consumed relative to sucrose was greatest at 4 h into the choice phase, so this could be an optimally sensitive monitoring period. The time at which withdrawal-induced drug seeking peaks could then guide work that characterises nicotine withdrawal symptoms in bees. This could perhaps be achieved using manual behavioural scoring (Hurst et al. 2014) or computer vision methods.

Is this a coevolved trait?

Ultimately we want to know if nectar drugs are a coevolved trait used to make addicts of pollinators. All of this is therefore only relevant insofar as bees can become addicted to natural nectar drugs at natural concentrations in natural contexts.

My literature search revealed that relatively little is currently known about the distribution of these compounds in nectar. Some targeted nectar sampling could be a useful starting point, perhaps with a focus on nAChR agonists to begin with. These concentrations may inform future behavioural experiments. Alternatively, these could be conducted using concentration series and later one could assess how the optimally addictive synthetic nectars relate to observed field concentrations. When collecting these data it may also be useful to sample the accompanying nutrients, as the rewarding and pharmacological effects of nectar may interact to determine how learned preferences arise (Francis et al. 2019, Hemingway et al. 2024).

The experimental protocol I developed to track bee food choice over time will now permit us to identify whether these nectars are addictive to bees, and my preliminary work shows that certain compounds do have this potential. For the sake of field relevance, simplicity, and sensitivity to emergent effects, if I could redo the latter experiment presented in chapter 4 I would monitor the patterns of voluntary drug interactions made by naive bees over longer timescales rather than subjecting them to three days of forced pre-exposure.

Using comparative phylogenetic approaches, it should be possible to identify whether several plant lineages have independently evolved to manipulate their pollinators with drugs. This would require the collection of data on the taxonomic distribution of nectar drugs as well as the associated nectar volume and sugar content, floral morphology, pollinator guilds, and their tissue distributions within plant species.

Conclusions

Nicotine-laced food is potentially addictive to bees because it can pharmacologically elicit pronounced though transient feeding preferences under *ad lib* conditions. Some individuals may have been more susceptible to this effect than others. Specific changes to learning and memory were assessed but remain unclear and could do with further study.

Nicotine pre-exposure increased voluntary drug uptake, and this nicotine-seeking was enhanced under withdrawal. Drug-deprived bees responded during conditioning in a way that suggests increased valuation of nicotine-laced food and undervaluation of undrugged food, and they showed some indication of stress and cognitive impairment. In sum, it is likely that bees in these assays had become addicted to nicotine and arecoline but this finding would ideally be confirmed by testing for self-administration in the presence of costs.

Chemically, it appears that these effects could be highly dependent on dose, mechanism of action, and molecular structure. However, we now have the tools to investigate whether addiction to these and other drugs may develop in the field.

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