The variability and adaptive value of visual learning performance in wild and domesticated colonies of the common eastern bumblebee, Bombus impatiens

by

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THE VARIABILITY AND ADAPTIVE VALUE OF VISUAL LEARNING PERFORMANCE IN WILD AND DOMESTICATED COLONIES OF THE COMMON EASTERN BUMBLEBEE, BOMBUS IMPATIENS

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This thesis examined the adaptive value of visual associative learning in the bumblebee Bombus impatiens, first by quantifying variation in performance across four laboratory colour-learning tasks for wild and domesticated colonies, and then by assessing the field foraging performance of individuals relative to their learning ability. Domesticated colonies differed significantly in terms of Learning Performance Index (LPI) in learning tasks. There was no evidence that colony LPI values were distributed differently in wild and domesticated populations. All colonies displayed similar behavioural patterns across learning tasks: learning performance was greatest when blue was the rewarded floral colour, and performance was significantly reduced after two reversals of the colour-reward association, indicating interference. Bees did not learn to prefer a novel, highly rewarding colour (green). In the field, bees with superior LPI scores collected significantly more pollen and nectar per foraging bout, but had similar overall productivity to bees with worse scores.
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Chapter 1: Introduction and literature review

1.1: General introduction

The relationship between insects and flowering plants comprises an ancient and complex web of interactions continuously driving evolution in both groups. Insects are by far the most ecologically important group of animal pollinators, and have acted as such since at least the Cretaceous (Grimaldi 1999). The extent to which these long-standing associations have contributed to speciation in its participants is unclear, but the fact remains that angiosperms and insects are currently the most taxonomically diverse groups of plants and animals respectively (Suchan & Alvarez 2015). As such, the foraging environment for flower-visiting insects today consists of a vast array of floral types, each offering rewards of differing quality and quantity, and each identifiable via a suite of sensory signals such as olfactory cues, shape, colour, texture, and electrostatic charge that may be more or less perceptible to different taxa (Dobson 2006, Reverté et al. 2016, Moyroud & Glover 2017). In this “floral marketplace,” the flowers that an insect chooses to visit can contribute directly to its net intake of energy and nutrients, as well as its risk of predation or pathogen infection (Moret et al. 1998, Chittka & Raine 2006, Gonçalves-Souza et al. 2008, McArt et al. 2014), factors that in turn affect fitness. Studying these flower choices, the external factors that inform them, and the behavioural patterns they represent has formed a large part of our current understanding of how learning and memory operate in insects.
1.1.1: The ecology and evolution of insect learning in the floral marketplace

Efficient foraging allows flower-visiting insects to achieve an energy surplus that can facilitate growth, reproductive function, and survival during times of scarcity, thereby contributing to fitness (Pyke 1984). Eusocial insects can derive similar fitness benefits from foraging efficiency, though these do not manifest at the level of individual foragers but rather at the colony level, as surplus energy can be used to produce additional sexual forms during colony reproduction (Pelletier & McNeil 2003, Seeley & Mikheyev 2003). It follows that in environments where resource availability varies spatially or temporally according to discernible patterns, it is possible for insects to make informed foraging decisions that are “correct” or “incorrect” from a fitness standpoint (Stevens 1991). On the other hand, in environments that either do not vary greatly over short timescales or vary randomly, decision-making based on available information will have negligible fitness benefits, as the information itself will provide little value with regards to the energy payoff of a given foraging decision. Floral marketplaces almost always represent the variable yet somewhat predictable type of environment where information is valuable, as the flowering phenology of most angiosperms is predictable through time and space. The spatial arrangement and composition of flower patches (Burns & Thomsoon 2006, Seifan et al. 2014), the succession of flowering species in these patches over the course of the growing season (Petanidou et al. 2008), as well as the opening, closing, depletion, and repletion of individual flowers (Goulson 1999, Puterril et al. 2004, Fründ et al. 2011) over much shorter timescales all represent patterns that can be detected, stored in memory,
and to a certain extent predicted. With this knowledge, one can assume that
flower-visiting insects will be selected for cognitive abilities that allow them to
optimize their foraging decisions, minimizing energy expenditure while maximizing
energy acquisition for each foraging bout. After decades of behavioural research,
the general consensus is that cognitive adaptations to the floral marketplace are
essentially omnipresent in flower-visiting insects (Dukas 2008, Avarguès-Weber et
al. 2010, Jones & Agrawal 2017). However, very little is known regarding the
application of these adaptations in the field, and whether they are a product of
continuous selection for improved foraging efficiency as theory suggests.

Although beetles (Coleoptera) and flies (Diptera) are frequently found on
flowers and in some cases act as pollinators (Weiss 2001), the great majority of
published work on flower-visiting behaviour is focused on bees (Hymenoptera),
butterflies, and moths (Lepidoptera). The ability to learn associatively is nearly
ubiquitous in these groups, at least in the context of flower foraging decisions. A
few studies have reported no learning during the host searching and oviposition
behaviour of certain hymenopteran and lepidopteran species (Parmesan et al.
1995, Potting et al. 1997, Kerpel & Moreira 2005), but without an examination of
their floral visitation behaviour it would be imprudent to assume that these species
eschew learning altogether. The conventional wisdom that associative learning is
of adaptive value in a floral marketplace has yet to be contradicted, but next to
nothing is known about the selection pressures that have driven—and could
continue to drive—the elaboration of learning ability in flower foragers. While
learning itself may be a universal property of these insects, the speed, precision,
and complexity of said learning varies greatly across taxa, even between species
that are either closely related (Qin et al. 2012, Wang & Tan 2014) or occupy the same environmental niche (Kandori et al. 2009). There can also be considerable heritable variation in cognitive traits between and even within populations of the same species (Chittka et al. 2004, Snell-Rood & Papaj 2009, Couvillon et al. 2010 Rodrigues 2016). Investigating the underlying causes of this variation will reveal the most important agents of selection on cognitive traits and also facilitate the prediction of insects’ cognitive responses to environmental change, both pressing issues in the study of behavioural evolution and ecology.

1.2: Agents of selection on insect learning ability

1.2.1: Variation in local environments

Over time, several theories have been put forward to explain observed differences in the cognitive aptitude of insect that forage on flowers. The first of these deals with characteristics of the foraging environment. Beyond the broad environmental factors that determine whether or not learning should be favoured in an insect, there should be variation on a finer scale in the floral marketplace informing the strength of selection. For example, environments that vary appreciably between generations but exhibit reliable sensory cues within generations are expected to select more strongly for learning ability than environments with little inter-generational change (Morand-Ferron 2017). The same reasoning can also be applied to the type of learning selected for: in the case of a local environment where floral rewards are generically plentiful across most plant species, but the flower patches themselves are separated by large distances
or complex physical barriers, one might expect insects to be selected for strong spatial memory and the ability to navigate to patches, with relatively little selection for strong discrimination of flowers based on colour, odour, or shape (Waser et al. 1996). While relatively simple to grasp, this type of hypothesis is extremely difficult to test due to the high number of environmental variables that need to be controlled or accounted for in a manipulative study. Greenhouse assays (Snell-Rood & Papaj 2009) and large outdoor flight cages (Makino & Sakai 2004, Lihoreau et al. 2016) have been tentatively explored as an approach to environmental manipulation, but for the most part investigations of local complexity as an agent of selection on learning have been limited to observational studies (Raine & Chittka 2008, Ings et al. 2009). As a result, it is widely accepted that environmental variability does exert selective pressure, but without a focus on specific environmental factors and their interactions with specific organismal traits, it is not currently possible to draw conclusions beyond this generality.

1.2.2: Physiological constraints in systems necessary for learning

In addition to the quality of their environment, one must also consider the innate characteristics of flower-visiting insects themselves as partial determinants of cognitive ability. The most straightforward of these traits are physical constraints such as size. Simple associative learning is a highly conserved property of the insect brain, but the relatively small number of neurons within necessitates the use of simple, compact circuits for storing memory (Giurfa 2013) and may even impose a hard limit to the development of higher-order cognitive functions (Adamo 2016). Given the extreme range of body and brain sizes exhibited between–and
occasionally within (D'Amico et al. 2001, Mares et al. 2005, Gronenberg & Couvillon 2010, Riveros & Gronenberg 2010)—flower-visiting species, it is possible that smaller species or individuals are handicapped by the simplicity of their neural networks, and are less likely to develop more advanced abilities demonstrated by their larger counterparts. While it is true that the most advanced cognitive functions found in insects to date have been reported from larger pollinators (Schubert et al. 2004, Giurfa 2013, Sommerlandt et al. 2014b, Loukola et al. 2017, Chittka 2017), this is at best anecdotal evidence and does not preclude the possibility of minute insects possessing these abilities. It must be noted as well that brain size and density may increase as a plastic response to adult experiences, as has been demonstrated for butterflies (Snell-Rood et al. 2009, Montgomery et al. 2014) as well as both social (Maleszka et al. 2009, Tomé et al. 2014) and solitary (Withers et al. 2008) bees. As cognitive traits that were once considered exclusive to vertebrates continue to be discovered in honeybees and bumblebees, it is becoming apparent that the structure of neural circuits may contribute more to an animal’s behavioural repertoire than the size and quantity of neurons (Chittka & Niven 2009, Giurfa 2013). Large-scale comparative studies that account for not only neuron count, but also different neuron types and the number of internal linkages (Chittka & Niven 2009, Perry et al. 2017) are necessary to properly evaluate the hypothesis that size is a true constraint in the evolution of learning.

Insect sensory systems comprise another trait that possibly constrains cognitive adaptation, as they determine the type and quality of raw information that can be stored in memory. For an insect to learn differences between stimuli within a sensory modality, it must possess not only pre-adaptations for processing the
appropriate level of resolution in that modality, but also neurological circuitry capable of forming separate associations at that level. Although recent investigations have revealed tactile (Alcorn et al. 2012) and electrostatic (Clarke et al. 2013) floral cues as likely influences on insect flower choice, discussion here will be limited to learning in the most well-studied and well-understood sensory modalities: vision and olfaction. Insects may value one of these modalities over the other when weighing information depending on their distance from flowers, available light levels, or the presence of conspecific individuals (Balkenius et al. 2006, Sanchez et al. 2011, Sommerlandt et al. 2014a), but they will inevitably make use of both over the course of their foraging routines (see Goyret et al. 2009, Leonard & Masek 2014).

The physiological underpinnings of colour vision are highly conserved across flower-visiting insects in the form of UV, blue, and green photoreceptors within their ommatidia, although many lepidopteran species possess red receptors that are largely absent from the Hymenoptera (Briscoe & Chittka 2001). Most of the floral displays found in nature can be distinguished from one another using this trichromatic vision, as one would expect given the general consensus that angiosperms have evolved to discourage interspecific pollen transfer and enable flower constancy from pollinators (Chittka & Menzel 1992, Chittka 1997). While many species of flower visiting insects display innate preference for specific floral colours, these preferences are not fixed and do not preclude the possibility of learning an attraction to other colours (Waser et al. 1996). Variation in preferences across species may represent adaptation to local flowering plant assemblages (Raine et al. 2006a, Raine & Chittka 2007), or simply the colour bias inherent to a
visual system with few receptor types. Regardless, variation in visual learning ability is ultimately a product of the neural machinery that determines how an insect evaluates optical information. More advanced forms of visual processing, such as storage of multiple discrete colour-reward associations or discernment of similar colours within the same photoreceptor type, have been reported from honeybees and bumblebees (Dyer & Chittka 2004, Dyer et al. 2011, Rohde et al. 2013, Muth et al. 2015b), but are generally less well-studied in other flower-visiting taxa. At present it is unknown if complex visual processing circuits are as highly conserved in these insects as the sensory equipment that enables them.

The general olfactory capabilities of flower-visitors are not as well understood across hymenopteran and lepidopteran taxa, at least not in terms of the sensitivity of their olfactory receptors to specific odorants. The responsiveness of insect antennae to various scent compounds can be quantified via electro-antennographic detectors, but considering the enormous diversity of flower-visiting insects, and the fact that nearly 2000 floral volatile compounds have been described so far (Knudsen et al. 2006), only a relatively small number of insect-odorant combinations have been tested with this technology. Fortunately, the honeybee *Apis mellifera* is one of those species that has been relatively well studied with electro-antennography (Dötterl & Vereecken 2010), and there is cause to believe that its range of olfactory perception is similar to those of other generalist flower-visitors. Honeybees exhibit antennal responses to all of the floral volatiles produced by several species of plants from different biomes (Henning & Teuber 1992, Pham-Delège et al. 1997, Twidle et al. 2015), and are not only capable of distinguishing between different compounds, but also different blends of multiple
compounds, and even between blends with different ratios of the same active ingredients (Getz & Smith 1987, Gurrieri et al. 2005, Wright et al. 2008). Study of the floral volatiles produced by angiosperms suggests that flowers are generally not recognizable by totally unique complex scent compounds, but rather by a distinctive bouquet of several compounds, the ingredients of which tend to have some overlap between plant species (Wright & Schiestl 2009, Dötterl & Vereecken 2010). All of this information suggests that the relative abundances of scent compounds may be more important to interspecific scent distinctions than their chemical structure (Pichersky & Lewinsohn 2011, Riffell 2011). Since most plant-pollinator networks tend towards generalization and redundancy of interactions (Waser et al. 1996, Bascompte & Jordano 2007), it is likely that many or all flowers in a given marketplace will fall within the olfactory range of perception of any foraging insect, at which point the insect’s ability to judge the aerial concentration of specific compounds becomes the most important for decision-making. Thus, much as with visual signals, hard perceptual limits are unlikely to be the main factor explaining observed differences in olfactory learning ability between flower-visiting insects, with the neural circuits required for storing sensory inputs in memory apparently playing a more important role. As before, most of the more detailed research into these cognitive features has focused on honeybees and bumblebees (Mota & Giurfa 2010, Andrew et al. 2014).

1.2.3: Life history traits as preadaptations for cognitive development

An insect’s life history, the intrinsic details of its physiology and life cycle, can enable or constrain cognitive adaptation, as can it’s ecological niche, the
behavioural axes along which it interacts with the physical environment. For instance, it has been argued that generalist flower foragers will be more strongly selected for learning ability than specialists due to the wider range of floral cues they interact with in the wild (Jones & Agrawal 2017). Specialists may also possess innate behavioural routines for finding and accessing the flowers for which they are adapted, granting an adaptive advantage over inexperienced generalists foraging on the same plants while reducing the need for learning ability and any biophysical costs associated with it (Chittka & Laverty 1988, Dötterl & Schäffler 2007). On the other hand, there may be times in nature where flowers that are attractive to specialists are in relatively short supply, incentivizing supplementary foraging on other species and maintaining selection for a more cognitive flexibility than might be expected from a specialist (Williams 2003, Riffell et al. 2008). It must also be noted that a specialist insect can encounter considerable phenotypic variation even within its preferred flowering species, creating situations where accurate learning is still favoured. Research into this particular ecological situation has been limited, but so far some specialists have proven capable of learning within-host variation, demonstrating the ability to associate floral visual phenotypes with reward quality and adjusting their visitation behaviour accordingly (Lewis & Zenger 1983, Weiss 1997). However, other specialists have failed to demonstrate this capacity under test conditions (Brandenburg et al. 2012, Oliveira et al. 2015), and the extent to which it occurs in nature remains an open question. It appears that the specialization-generalization gradient does not provide a hard and fast rule for the evolution of learning, but is nevertheless a factor to be considered when making predictions about an insect’s cognitive abilities.
Sociality, a characteristic of several flower-visiting hymenopteran species, is another aspect of life history that has been proposed to facilitate cognitive evolution. The reasoning is that a “social brain” will possess a number of pre-adaptations for more advanced learning and memorization due to the unique cognitive challenges posed by sociality (Lihoreau et al. 2012a). Social environments might grant adaptive value to behaviours requiring cognitive-like processes, such as nestmate recognition, task specialization, and advanced forms of communication. It is particularly important for large eusocial colonies that nestmates can be distinguished from intruders based on either chemical or behavioural cues, and honeybees and bumblebees make use of both forms of information when other bees enter their colonies from outside (Plowright & Fuller 1988, Couvillon et al. 2008). Some social wasps even display facial recognition ability (Sheehan & Tibbetts 2011, Baracchi et al. 2015). Workers may also be required to adjust their behavioural routines and switch between specialized tasks depending on the needs of the colony, whether this involves foraging for a specific type of resource, performing colony maintenance and brood care operations, or even acting as a guard or raider against other colonies (Free 1955, Moore et al. 1987, Johnson 2003).

Various forms of social communication have been documented in flower-visiting social insects as well, including the exchange of information on floral reward quality through trophallaxis (Farina et al. 2005, Molet et al. 2009), and the use of vibrational signals to recruit idle workers to internal colony tasks (Cao et al. 2009, Mc Cabe et al. 2015). The most well-known communicative behaviour is the remarkable waggle dance of the honeybee, used to communicate geometric
concepts such as angle and distance that are retained in memory (von Frisch 1967). A local-enhancement variety of social learning, whereby individuals learn a rewarded stimulus more quickly when it is accompanied by the visual and olfactory cues associated with conspecifics, has also been observed from bumblebees and stingless bees (Leadbeater & Chittka 2007, Sommerlandt et al. 2014a).

While it is not possible to observe changes in the brain of a solitary insect species transitioning to eusociality over an evolutionary timescale, there are species that display intermediate forms of sociality that could be treated as test cases for the “social brain” hypothesis. Study of the facultatively social bee *Megalopta genalis* has provided evidence that social behaviour stimulates solitary individuals to augment the development of their mushroom bodies, which are areas of the brain associated with sensory processing and learning (Smith et al. 2010). This observation makes a case for sociality as a driver of selection for advanced cognition, but more studies comparing the learning abilities of eusocial, semi-social, and solitary insects, both in terms of brain development and manifested cognitive abilities, are needed to properly evaluate this hypothesis. To date, there have been relatively few attempts to make such comparisons, and their results have been less decisive than theory would predict, with solitary bees falling behind their social counterparts in certain learning tasks but performing as well or even better in others (Dukas & Real 1991, Amaya-Márquez & Wells 2008). There are also differences in learning ability between eusocial species (Mc Cabe et al. 2007, Qin et al. 2012, Moreno et al. 2012, Henske et al. 2015) that cannot be explained by level of social complexity alone.
1.2.4: Potential fitness costs of learning

The aforementioned ecological factors may be selecting for learning ability in floral markets, but they may be more likely to be exerting stabilizing selection rather than simple directional selection, as there are certain drawbacks associated with greater cognitive ability that could on occasion outweigh its benefits. It is generally accepted that increased investment in neural tissue is energetically costly, so the enlargement of brain areas like the mushroom bodies that are associated with learning and memory may place greater metabolic demands on an insect and affect its activity levels or reproductive potential (Mery & Kawecki 2004, Snell-Rood et al. 2011). Direct costs of this type may be global in insects that exhibit strong learning and possess well-developed brains from their first emergence as adults, or induced in cases where insects exhibit additional neurological development in response to cognitive challenges faced post-emergence (Fahrbach et al. 1998, Mery & Kawecki 2004, 2005, Withers et al. 2008, Snell-Rood et al. 2010). While the latter situation mitigates the absolute cost of learning, there will likely be some reduction in fecundity and survival in both cases, albeit not enough to undermine the fitness benefits incurred and reverse the direction of selection. There may also be pleiotropic effects tied to selection for learning ability if the genes involved in regulating neurological development are linked to other aspects of an insect’s phenotype. Although the genetic architecture underlying insect behavioural patterns is not well understood outside of Drosophila model systems, studies focusing on honeybees have demonstrated that loci responsible for regulating foraging overlap with several other regulatory systems, including those associated with nutrition and reproductive development (Rüppell et
al. 2004, Grozinger et al. 2007, Ament et al. 2008). Correlation between reproductive status and learning ability has been reported in bumblebees (Evans et al. 2016), and given that the genetic distance between honeybees and bumblebees is relatively small (Stolle et al. 2011), it is possible that pleiotropic effects are in play for *Apis* species as well. Further studies of fitness costs incurred by enhanced learning performance will be valuable for explaining discrepancies in insect learning ability, especially in the case of intraspecific or, in the case of social species, inter-colony variation.

Understanding the evolution of learning in flower-visiting insects is no easy task, and it is likely that all of the agents of selection discussed here have played a role in shaping the cognitive processes currently on display. The difficulty that behavioural ecologists face lies in evaluating these hypotheses by making connections between learning ability and fitness under natural conditions. To that end, current methodologies for tracking insect behaviour and performance in the laboratory and the field must be developed further.

### 1.3: Methods for quantifying learning performance in flower-visiting insects

Since evidence of associative learning was first discovered in insects, researchers have proposed and tested numerous experimental paradigms in response to the question of how best to empirically test the learning abilities of pollinators. A major difficulty in designing a protocol for detecting cognitive faculties lies in the fact that they must be detected indirectly, by making inferences based on observations of an organism's behaviour (Mery 2013). Another major issue lies with the extreme variation in size, functional morphology, and flight behaviour of
flower-visiting insect species, which prevents the adoption of standardized materials, methods, and measurements that can be applied to the entire group. For any cognitive testing protocol to be of value, it must be easily replicable, tailored to the physiology and life history of the study organism, and capable of distinguishing cognitive processes from similar non-cognitive mechanisms such as sensitization (Papaj & Prokopy 1989) or innate behavioural rules (Keasar et al. 1996). The many protocols that have been developed to date that fit this description rely on testing a small number of well-established assays and model organisms, and can be broadly separated into two categories depending on whether the main objective is detection or proficiency analysis.

1.3.1: Protocols for detection of cognitive faculties

The oldest and simplest of behavioural testing protocols are those which are only concerned with whether or not an insect possesses a specific cognitive ability. Response variables within this experimental framework can be divided into three discrete categories, depending on the subject organism’s behaviour: it either displays the ability, does not display the ability, or fails to respond to the protocol altogether. Many of the more advanced tests in use today trace their roots back to this type of assessment. For example, the earliest applications using the innate proboscis extension reflex (PER) to sucrose as a behavioural assay of associative learning in bees relied upon classical conditioning (Takeda 1961, Bitterman et al. 1983). This allowed for the discovery of olfactory discrimination and learning in bees. A similar classical conditioning paradigm using colours was the means by which colour vision and learning were first discovered in bees (von Frisch 1914).
Visual and olfactory learning tasks for insect subjects have developed considerably from these basic roots, but the simplest type of detection protocols can still be useful when testing for learning and memory in previously un-researched species (Fukushi 1989, Lunau & Wacht 1994, Amaya-Márquez et al. 2008, Mc Cabe & Farina 2010, Sánchez & Vandame 2012, Henske et al. 2015) or when testing for discrimination ability in a newly discovered sensory modality such as electromagnetic field sensing (Clarke et al. 2013).

Once a baseline of cognitive ability has been established, it becomes possible to search for more advanced faculties by adding new levels of difficulty to the basic behavioural assays. Incorporating compound stimuli into the traditional PER conditioning protocol has allowed researchers to test for various forms of non-elemental olfactory learning, discovering that honeybees are capable of negative pattern discrimination (Deisig et al. 2001) while bumblebees appear unable to do so (Sommerlandt et al. 2014b). In the same vein of research, adding additional stimuli and rewards to established colour learning tasks precipitated the discovery that bumblebees (Fauria et al. 2002, Worden et al. 2005, Muth et al. 2015b) and butterflies (Weiss & Papaj 2003, Cepero et al. 2015) are capable of learning and contextualizing multiple associations simultaneously, albeit with some interference. Combining olfactory and visual stimuli in learning tasks is another way for researchers to increase complexity, and has revealed that multimodality of stimuli reinforces associative learning in bumblebees (Lawson et al. 2017), while hawkmoths only learn associations in a single sensory modality at a time (Balkenius et al. 2008, Balkenius & Dacke 2013). Protocols for testing spatial and sensorimotor learning in insects are considerably less standardized across studies,
but follow the same principle of starting with a simple task and increasing the complexity to determine the limits of a subject’s absolute capabilities. This approach led to the discovery of new qualities such as handedness (Cheverton 1982, Kells & Goulson 2001) and scaffold learning (Mirwan & Kevan 2014, Loukola et al. 2017) in the cognitive architecture of bumblebees. As more species are investigated and additional cognitive tasks are devised, protocols for detecting cognitive abilities, whether basic or advanced, will continue to be used at the frontiers of behavioural ecology.

1.3.2 Protocols for measuring relative proficiency in cognitive tasks

Understanding what cognitive tasks an organism is capable of can be useful for comparing different species, but higher resolution is necessary for any examination of intraspecific cognitive variation. It is unlikely that conspecifics will be so neurologically diverse as to possess completely different abilities, but they may still differ in their levels of competency due to a combination of ecological and evolutionary factors. In order to investigate these factors and their influence on insect cognition, accurate measures of relative performance within a cognitive task are essential. Acquisition time, error rate, and saturation performance are all measures that are commonly used in both conditioned PER assays and free-flying visual learning tasks with artificial flowers or mazes (Raine et al. 2006b, Smith & Raine 2014). Average and peak problem-solving speed are also useful measures of proficiency, particularly for subjects that are required to complete a sensorimotor task to access rewards (Laverty 1994, Gegear & Laverty 1995). In studies of traplining or other route optimization strategies of free-flying insects, distance
traveled per foraging bout is a functional indicator of performance, and can be used in conjunction with known reward levels to estimate a subject’s net energy gain per bout (Waddington 1985, Saleh & Chittka 2007, Lihoreau et al. 2012b, 2012c). Comparative measures like these have enabled researchers to isolate the negative cognitive effects of factors such as pathogen infection (Gegear et al. 2005, Otterstatter et al. 2005, Gegear et al. 2006) and exposure to pesticides (Decourtye et al. 2005, Stanley et al. 2015) on social bees, both being important issues for pollinator conservation (González-Varo et al. 2013, Vanbergen & Initiative 2013). The cognitive impact of other ecologically relevant factors that are known to affect worker activity, such as intraspecific variation in body size (Mares et al. 2005, Raine et al. 2006b) and nutritional stress (Mattila & Smith 2008, Scofield & Mattila 2015) have also been studied in this way, though significant positive or negative interactions with learning and memory have yet to be detected.

1.3.3: The future of cognitive testing protocols

All of the applications of comparative cognitive measurements discussed in this section have furnished valuable contributions to the literature of insect cognition, but they also share the common limitation of being largely divorced from the ecologically realistic conditions under which most selection takes place. It is certainly useful to understand how specific factors affect insect learning performance in a controlled laboratory environment, but for far-reaching ecological theories to be built and tested, and for policy decisions to be properly informed, it is important that the operation of insect cognitive traits be observed in a natural floral marketplace. Bridging the gap between traditional studies of learning and field
studies in which fitness is measured has proven to be incredibly challenging, due mainly to the difficulty inherent in observing winged insects in open environments over extended periods of time. In recent years there have been some attempts to study the importance of learning in field settings, but even these have been forced to make some sort of compromise in their methodology. Free-Moving Proboscis Extension Response (FMPER) has recently been introduced as a less disruptive form of PER conditioning that can measure learning for wild bees in the field, but even this method still requires capturing individuals and confining them in small tubes for over an hour (Muth et al. 2018), and cannot immediately be associated with measures of fitness. On the other hand, if a study focuses mainly on fitness proxies such as pollen and nectar collection rate, inferences can only be made about learning having occurred based on whether these measures increase over time or not (Gill & Raine 2014). Another challenge in outdoor environments, or even in laboratory experiments with animals taken from the field, is that it is impossible to account for an organism’s experiences prior to cognitive measurement unless organisms are reared and confined under laboratory supervision where all experiences from naivety onward can be controlled (Morand-Ferron et al. 2016). Until monitoring technology becomes sufficiently advanced to track the entire decision-making process of flower-visiting insects in natural environments, the most effective and informative protocol is likely to be a combination of laboratory and field methodologies: the learning ability of individuals can be assessed using traditional protocols in an environment with minimal confounding factors, after which the individuals are released into the field where their foraging performance and survival can be observed. This type of experiment
would not necessarily be suited to the life cycles and foraging strategies of all flower-visiting insects, but fortunately it is at least applicable to well-established experimental subjects such as bumblebees, which have already received considerable attention in both laboratory and field behavioural monitoring protocols (Raine & Chittka 2008, Evans et al. 2017).

1.4: Bumblebees as a model system for the study of insect learning

The genus *Bombus*, comprising over 250 species of bumblebees widely distributed across much of the planet (Williams 2005, Michener 2007), is extremely well-represented in the literature of insect cognition. There are several explanations for this, not the least of which being simple availability. Due to their large size and distinctive flight patterns, nest-searching bumblebee queens are generally highly conspicuous and easy to catch, and since the turn of the 20th century there have been numerous documented attempts at rearing colonies from such queens of several different *Bombus* species (see Sladen 1912, Plath 1923, Frison 1927, Hasselrot 1952, Plowright & Jay 1966, Pomeroy & Plowright 1980, Yeninar et al. 2000, Yoon et al. 2002, Strange 2010). Even more important, though, is the fact that certain species have been successfully cultured in captivity to the point of being commercially domesticated (Velthuis & van Doorn 2006). As a result it has been possible for researchers to acquire multiple bumblebee colonies from commercial suppliers for over two decades, allowing for the difficulties of the rearing process to be avoided entirely. This is reflected in the literature, with most behavioural studies focusing on the domesticated species *Bombus terrestris* in Europe and *Bombus impatiens* in North America.
Colony development and rearing are not the only aspects of bumblebee biology that have been thoroughly studied. Their physiology, social organization, and foraging strategies are also relatively well-understood which opens up numerous possibilities for focused studies of highly specific aspects of their behaviour. Bumblebees are spread out across the generalization-specialization gradient, with representatives ranging from polylectic short-tongued species to oligolectic or even monolectic long-tongued species (Goulson & Darvill 2004, Jiaxing et al. 2015). It is therefore possible to study the effect of food specialization on the evolution of learning by comparing these relatively closely-related species (Laverty & Plowright 1988). The primitively eusocial colony structure displayed by all non-parasitic *Bombus* species is another factor that can be explored in the context of behavioural studies. Social transmission of learned information among bumblebees has been tested in visual (Avarguès-Weber & Chittka 2014), olfactory (Leadbeater & Chittka 2007), and sensorimotor (Alem et al. 2016) learning tasks, and the effects of caste differentiation and body size on learning have been documented (Worden et al. 2005, Evans & Raine 2014a, Lichtenstein et al. 2015). Since there are established methods for manipulating ovarian development in bumblebee workers, it is even possible to assess learning at different points in this developmental process (Evans et al. 2016). The large number of workers present in colonies essentially guarantees sufficient sample sizes in studies of this type, so intra-colony variation in learning can be quantified at high resolution. As central place foragers, bumblebees are also convenient subjects for field-based studies of foraging efficiency, as their nectar and pollen collection can be assessed when they return to the colony.
Even if it were less practical to work with bumblebees, there would still be considerable economic and environmental incentives to study their biology and behaviour. Since becoming commercially domesticated, these bees have become preeminent pollinators of greenhouse crops, outperforming honeybees due to their superior navigation of indoor environments and ability to dislodge pollen through sonication (also called buzz pollination: Paydas et al. 2000, Switzer & Combes 2017). Understanding the cognitive processes behind their foraging patterns might allow for their pollination efficiency, and by extension yield, to be further increased. Wild bumblebees also provide valuable pollination services to flowering plants in agricultural and natural systems alike, and are considered among the highest priority targets of pollinator conservation efforts (Goulson 2003, Goulson et al. 2008). Investigation of the cognitive traits of bumblebee species and how they interact with global environmental change is an important aspect of these efforts. Preliminary efforts have been made to study the importance of learning to bumblebees foraging in natural environments (Raine & Chittka 2008, Evans et al. 2017), but these have been limited to the European *B. terrestris* and have produced results suggesting different fitness effects of learning ability at colony and individual levels. In order for the nature and directionality of these effects to be clarified, further studies of bumblebee learning and foraging efficiency are needed.

1.5: Conclusion

Associative learning is nearly ubiquitous in flower visiting insects, and impacts the fitness of both these insects and the plants they pollinate. Interspecific and intraspecific variation in learning ability has been observed in flower visiting
insects, provoking the development of numerous ecological hypotheses pertaining
to the evolution of cognition. Until recently it was not possible to test these
hypotheses under ecologically realistic conditions, but study protocols have now
advanced to the point where this goal is attainable. The following chapters describe
the application of established protocols for measuring learning ability and foraging
efficiency to an ecologically relevant model organism, the common eastern
bumblebee *B. impatiens*. The first goal of this investigation is to quantify variation
in learning performance across multiple learning tasks within and across colonies,
as well as between wild and domesticated populations of *B. impatiens*. The second
is to determine if there is a relationship between learning ability and foraging
performance for individual bumblebees in the field.
Chapter 2: Captive rearing of wild *Bombus impatiens* queens and visual learning assessment with wild colonies

**Abstract:**

As behavioural studies of *Bombus impatiens* have almost exclusively used commercial colonies, the cognitive abilities of wild colonies remain virtually unknown. Wild colonies, unlike domesticated colonies, are representatives of the species that are experiencing natural selection for cognitive traits, and investigation of these traits is more likely to reflect how *B. impatiens* behaves in nature than experiments with commercial colonies. In this chapter, methods are presented for successfully rearing colonies of *B. impatiens* from wild-caught queens, and for carrying out behavioural experiments on said colonies using protocols that are more commonly used on commercial colonies. Naïve workers from the two mature colonies reared were subjected to four serial colour learning tasks while foraging on artificial flowers in a flight arena: (1) a task that determines how quickly they learn to associate the colour yellow with floral rewards, overcoming their innate preference for blue; (2 & 3) two sequential reversals of the colour-reward association in this task; and (4) a final task that supplemented the original foraging scenario with a novel, highly-rewarding flower colour. Learning Performance Index (LPI) scores were generated for each bee based on the number of errors (non-rewarding flower choices) made during completion of each task. Bees learned to associate blue with nectar rewards faster than they did yellow. They also showed signs of interference effects resulting from reversal of the colour-reward association, as their learning performance in the second task with rewarding yellow
flowers was significantly worse than in the first. None of the colonies studied showed any learned preference for the novel, highly rewarding flowers introduced in the final task.

2.1 Introduction:

Variation in insect behavioural traits, whether innate or learned, is a feature of many insect populations, with genetic underpinnings that are beginning to come into focus as a result of studies on *Drosophila melanogaster* (Bendesky & Bargmann 2011). However, domesticating insects and breeding them in captivity can result in genetic divergence from wild populations, including differences either in behavioural traits or the variation in these traits (Boller 1972). Studies of the European *Bombus terrestris* have revealed that wild bumblebee populations can differ in terms of their learning speed and innate colour preferences, as well as the strength of these preferences (Chittka *et al.* 2004, Raine *et al.* 2006a, Raine & Chittka 2007, Ings *et al.* 2009). These qualities in turn inform their flower visitation behaviour and the cognitive challenges they face while foraging. The same degree of behavioural variation may not be present in commercial populations, which are not exposed to the same environmental selection pressures as wild ones. Instead, commercially reared bumblebees display signs of anthropogenic selection, either intentional or inadvertent, for larger colonies with greater reproductive activity (Ings *et al.* 2006, Inoue *et al.* 2012). Considering that *B. impatiens*, like *B. terrestris*, is a widespread generalist bumblebee that has been domesticated for several decades, it is likely that the wild and commercial populations of this North American species have also diverged in some behavioural or ecological traits.
In the wild, *B. impatiens* is a highly abundant pollinator with a large number of documented ecological linkages found throughout Eastern North America (Kearns & Thomson 2001, Lozier *et al.* 2011), but almost all current knowledge of its cognitive abilities and pollination efficacy exists with reference to its status as a domesticated pollinator of commercial crops. Although there have been several studies of learning and memory in *B. impatiens* reared by commercial suppliers, similar studies of wild *B. impatiens* remain conspicuously absent from the behavioural ecology literature. A major cause of this knowledge gap is the difficulty and unpredictability inherent in rearing bumblebee colonies from wild-caught queens, a process that is not refined or well-understood outside of proprietary methods that are closely guarded by commercial bumblebee breeders and distributors. The same rearing methods can yield drastically different results depending on the *Bombus* species used, the materials used for nest boxes, the types of pollen available, and the timing and location of the rearing process itself (Plowright & Jay 1966, Génissel *et al.* 2002, Yoon *et al.* 2002). The ready availability of commercial colonies provides an appealing alternative for those operating under time and resource constraints. However, as there is reason to suspect that these domesticated bees are not entirely representative of their wild counterparts, it is difficult to draw ecological conclusions about the behaviour of *B. impatiens* based on studies of commercial colonies alone. In order to understand how the learning and memory capabilities of wild bees might be divergent from those of their domesticated counterparts, it is necessary to study them using the same the same cognitive testing methods that have hitherto only been applied to commercial colonies.
The first objective of this study is to establish a precedent for studying cognition in wild *B. impatiens* by rearing colonies from wild-caught queens using simple, easily reproducible methods, then by carrying out behavioural experiments on these colonies before they complete their life cycle. The objective of the experiments is to measure the speed and accuracy of visual learning by worker bees from wild colonies as they face different cognitive challenges, to create a point of reference for comparing their performance with that of commercial colonies of the same species. In accomplishing this, the degree of inter-colony and intra-colony variation in these qualities present in wild *B. impatiens* populations can also be better understood. This represents the first application of the visual learning protocol outlined in Raine *et al.* (2006b) to wild *B. impatiens*.

### 2.2 Materials and methods:

#### 2.2.1 Captive rearing of wild *B. impatiens* colonies:

Wild queens (*n* = 154) were collected through aerial netting from the University of Guelph Arboretum and main campus between May 3 and June 13, 2016. Netting took place on clear days between 8:00 h and 17:00 h, and care was taken to only capture queens that appeared to be in the process of nest searching—flying circuitously over open terrain without stopping to collect nectar or pollen. Each queen was confined in a 7.6 x 8.8 x 11.6 cm plastic container with multiple punctures in the lid for ventilation. Every container was provisioned with a feeder, made from a 10 mL oral syringe containing 30% (m/v) sucrose solution. Also included in each container was a pollen ball, a homogenized mixture of ground
frozen honeybee-collected pollen (Overton Apiary, Caledon, ON) and 30% sucrose solution, which acted as solid food for the queen and her brood. The defrosted pollen was manually crushed with latex gloves, then gradually moistened with sucrose solution using an eye dropper following the methods outlined in Kearns & Thomson (2001). Once enough sucrose solution had been absorbed to give the pollen a dough-like texture, the mass was rolled into a narrow cylinder and cut into pieces, which were then molded into the ~1.5 cm diameter balls that were used to provision nest containers. A small amount of blended honey was added to both the sucrose solution and the pollen ball incorporating an olfactory cue which facilitated discovery and ingestion of food by queens (Kearns & Thomson 2001). The provisioned containers were kept in a completely dark growth chamber with a portable heater and humidifier maintaining conditions of ~28°C and ~50% Relative Humidity. Pollen balls were replaced every other day unless the resident queen had laid eggs on them, in which case an additional, fresh pollen ball was added to the container. Sucrose solution feeders were rinsed out with water and refilled every three days, or whenever they were (nearly) empty. Any feces or mould near where the food and/or brood clump was located was scraped out of the container with a sharp edged blade. In order to reduce disturbance to the queens and their first broods, these maintenance operations were performed in the dark growth chamber under red light. Queens were allowed to lay eggs and develop their brood clumps under these conditions until the first adult workers eclosed. As soon as adult workers were detected in a container, the queen, workers, and any brood clumps were all transferred to a 30 x 20 x 12 cm wooden nest box. Each nest box was divided into two chambers of equal size: the front chamber contained a layer
of wood-based cat litter and provided a space for defecation and disposal of other waste, while the rear chamber contained the brood and food storage pots. Access to the front chamber was initially blocked following the transfer to prevent queens, workers, and brood from becoming separated during the critical early stages of colony founding. Provisioning continued following transfer of the young colonies, with the addition of a pollen ball approximately equal in size to that of the combined brood clumps every other day, and the simultaneous removal of any old, uneaten pollen balls. Once a second generation of workers had pupated and emerged as adults, access to the front chamber of the nest box was permitted. At this point, fecal samples were taken from the colony and examined under a compound microscope for evidence of infection by common bumblebee gut parasites that could cause behavioural impairment in the mature colonies during learning experiments. The main pathogens that were screened for were the trypanosome *Crithidia bombi*, the microsporidians *Nosema bombi* and *Nosema ceranae*, and the nematode *Sphaerularia bombi*, all of which can be visually identified in bee feces (Khan 1957, Otterstatter & Thomson 2006, Otti & Schmid-Hempel 2007). The process of provisioning and transferring the young colonies continued until every colony either established a stable population of workers or died out. Colonies with stable populations of more than 30 workers were then moved from the growth chamber to the laboratory for pre-training and learning experiments.
2.2.2 Pre-training foragers:

The cognitive traits of worker bees from the successfully reared wild colonies were assessed in the laboratory between 14 September and 6 October, 2016. Prior to testing, workers from each colony were pre-trained to carry out nectar foraging bouts on artificial flowers in an indoor environment. Each colony box was connected to a 120 x 100 x 35 cm flight arena with a transparent UV-transmittent Perspex® lid and a green card stock floor, and workers were allowed to forage freely on four bi-coloured artificial flowers. “Flowers” were squares with sides of 24 mm, half blue (Perspex Blue 727®) and half yellow (Perspex Yellow 260®), and were placed on top of 40mm tall glass collection vials to raise them off of the floor of the arena. Each flower was rewarded with a ~10µL droplet of unscented 30% sucrose solution that was immediately replenished with a pipette every time a visiting bee depleted it. In order to track the identities of experimental subjects across multiple bouts and trials, regular foragers were removed from the arena and marked with coloured and individually numbered tags (Opalith tags; Christian Graze KG, Germany). Tagged bees were then returned to the colony and allowed to resume their foraging activity. Workers that completed at least five foraging bouts in the pre-training phase were considered motivated foragers, and 15 such workers per colony were selected for cognitive testing. For each tested worker, cognitive testing began immediately following its last pre-training flight, and all tests were completed within an 8-hour period. Both pre-training and cognitive testing flights were illuminated by 32-Watt daylight (Colour Rendering Index = 90) fluorescent bulbs.
2.2.3 First forward learning task:

The cognitive experiment consisted of a set of serial colour learning tasks based on a well-established protocol described in Raine et al. (2006b). In the basic original form of the protocol, individuals had to learn to associate yellow artificial flowers with nectar rewards, overcoming their innate preference for unrewarding blue flowers in the process (Raine et al. 2006b, Raine & Chittka 2008). Once a foraging worker was selected for learning experiments, all other bees were excluded from the flight arena by a series of Perspex® gates for the duration of the four serial learning tasks. For the first colour learning task, 10 yellow and 10 blue mono-coloured artificial flowers were placed at stochastically distributed positions within the arena, based on where the collection vials that were used to elevate them were positioned after being dropped into the arena. These flowers were the same shape and size as the bi-coloured training flowers, but each had a central well that could either contain a sucrose reward or not (unrewarding). The well prevents flying bees from detecting sucrose solution droplets visually and discriminating based on this information, leaving flower colour as the only visual information to be associated with reward. In this task, yellow flowers were filled with a reward of 10µL unscented 30% sucrose solution, while blue flowers were empty (unrewarded). The bee was allowed to forage under these conditions until it completed five foraging bouts, or, in the case of bees that were observed making a large number of flower choices per bout, until it had made 100 flower choices after first landing on a rewarding flower. In this and all subsequent learning tasks, a flower choice was defined as a bee approaching or landing on a flower, and a
foraging bout was defined as any period of time between a bee leaving and reentering its colony during which flower choices were made. All flower choices were recorded to give a complete behavioural sequence and chart the increased choice frequency of yellow flowers as each bee formed learned associations between this colour and food rewards. To prevent depletion, scent marking, and positional memorization of flowers from affecting the bee’s decision-making across multiple bouts, all flowers were replaced with a new randomly-positioned set of 10 rewarding yellow and 10 empty blue flowers after the bee returned to its colony at the conclusion of every bout. After removal, all flowers were washed with detergent and water and then allowed to dry completely before being re-used in subsequent learning tasks.

2.2.4 Reversal learning task:

Each bee was presented with a reversal learning task immediately following completion of the first forward learning task. In this task the bee was presented with 10 rewarding blue flowers (containing 10µL of unscented 30% sucrose solution), while yellow flowers were empty (unrewarding). The sequences of all flower choices were recorded, and each bee was again considered to have completed this task once it made 100 flower choices after first landing on a rewarding flower, or completed five foraging bouts.

2.2.5 Second forward learning task:

Subsequently, the learning performance of each bee was assessed in a second forward learning task (rewarding yellow flowers and unrewarding blue
flowers) upon completion of the reversal learning task. Although it is referred to here as a 'forward' learning task, to the bee it represents a second reversal of an acquired colour-reward association, and allows for assessment of its cognitive response to such a serial-reversal of colour-reward contingencies. Except for this additional cognitive context, the task was methodologically identical to the first forward learning task.

2.2.6 Novel colour learning task:

In order to examine the response of bees to novel visual information, as well as their cognitive flexibility when required to repeatedly learn multiple colour-reward associations, the final learning task added a third (novel) flower colour and an additional level of food reward. After a bee finished the second forward learning task, it was allowed to forage in an arena containing 10 empty blue flowers, 10 rewarding yellow flowers (containing 10µL 30% sucrose), and 10 more highly-rewarding green (Perspex® Green 6205) flowers (containing 10µL of 45% sucrose), for a total of 30 flowers. As before, all flowers were replaced and refilled between foraging bouts. The task ended when a bee completed its fifth foraging bout or made 100 flower choices after first landing on a highly-rewarding green flower.

2.2.7 Video recording and behavioural scoring:

During each cognitive task, the movements of the subject bee were video recorded (with Sony CX220e and CX405 digital camcorders at standard definition). This method allowed for cognitive testing of multiple bees at once (in different flight
arenas), as decision-making and learning could be evaluated by reviewing video footage rather than scored through direct observation in real time. Each camera was fixed directly above a flight arena such that all artificial flowers used in a task were entirely visible within the frame. Recording began immediately before a bee entered the arena at the beginning of a task, and terminated once the task was considered complete according to the criteria outlined above (sections 2.2.3-2.2.6).

The sequence of flower choices made by each bee was determined through review of the video file for each task. A bee was considered to have made a flower choice whenever it approached or landed on a flower. Approach behaviour was defined as the bee altering its flight path towards a flower, reducing flight speed, and orienting its head towards the flower while coming within two body lengths of the flower. In the original paradigm that formed the basis for the four learning tasks administered here, the first instance of a bee probing a rewarding flower with its proboscis is regarded as the point where learning can begin, as it represents the moment a bee becomes aware of the positive colour-reward association. The choices made before this point are therefore treated differently than the choices made afterwards. However, the digital recording method used in the present investigation rendered probing events difficult to see on video, so the first time a bee landed on a rewarding flower was treated as the beginning of learning instead. Thus, for each bee in each cognitive task, the entire choice sequence prior to first landing on a rewarding flower was recorded, after which point only the next 100 choices were recorded. Learning ability was determined based on the reduction of erroneous (non-rewarding) choices over the course of this 100-choice sequence. In the case of the novel colour learning task that incorporated two reward levels, only highly-
rewarding green flowers were considered correct choices in order to evaluate whether bees learned to prioritize high-quality resources during foraging bouts.

2.2.8 Measuring learning performance:

The learning performance of individual bees was measured as Learning Performance Index (LPI), which is a single number summarizing the features of a bee’s “learning curve,” or the rate at which bees reduced the number of “errors” or non-rewarding flower choices made over the course of a 100 choice sequence. In order to calculate LPI values, these curves were fitted to each bee’s flower choice sequence data using the following equation for a first-order exponential decay function:

\[ y = y_0 + Ae^{-x/t} \]

where \( y_0 \) is the y-offset, \( t \) is the decay constant that determines the slope of the curve, and \( A \) is the amplitude. The \( x \)-value is the number of flower choices a bee has made in its progress towards 100 choices, and is a measure of both foraging time and experience. The \( y \)-value represents the rate at which a bee commits errors, in terms of how many errors are made per 10 flower choices. The \( y_0 \) value can be interpreted as the saturation learning performance of a bee, or the point beyond which it ceases to reduce its error rate. The \( t \)-value represents the speed at which a bee reduces its error rate, and can therefore be treated as a measure of learning speed. Since a bee cannot make more than 10 or fewer than 0 errors within a sequence of 10 flower choices both \( A \) and \( y_0 \) were constrained between 0 and 10 for the curve-fitting process.
Learning curves were fitted to 11 data points derived from a bee’s choice sequence, representing change in error rate over a period of 100 flower choices starting from first landing on a rewarding flower. At 0 choices, this rate was the proportion of errors made per ten flower choices before the bee first landed on a rewarding flower, a measure of the bee’s initial preference for unrewarding flowers. For bees that made fewer than 10 choices before landing on a rewarding flower, this value was set as the colony mean proportion. For each following data point, the number of errors made within each block of 10 choices over the next sequence of 100 choices was used. Curves were generated in Microcal Origin Pro 2017 software using the Levenberg-Marquardt fitting algorithm. The curves were then used to generate LPI values by summing the errors made by each bee at 5, 50, and 100 choices, as predicted by its learning curve function. The result was a number between 0 and 30, with lower values indicating better performance (fewer erroneous choices) and higher values indicating worse performance. LPI is a measure of two aspects of learning performance: saturation performance level and the speed at which an individual reaches this level of performance.

While the methods used here for generating learning curves are consistent with those established in the literature (Raine & Chittka 2008, Evans et al. 2017), the use of digital cameras for observing bees and the modification of the choice sequence scoring method necessitated by their use are unique to this investigation. This introduces a single disadvantage relative to the established protocol: the ability to determine when a bee first encounters floral reward is reduced as probing behaviour cannot be reliably detected. However, there is also the added advantage that the accuracy of the 100 choice sequence that follows
first probing should be greater, since the video recording can be paused, slowed down, and reviewed as many times as needed. The disadvantage inherent to this modified protocol would only outweigh the advantage of a persistent video record if the moment of first probing a rewarding flower was consistently and significantly mischaracterized by the video reviewer, causing the entire following choice sequence to be unrepresentative of the bee’s learning process. In order to determine whether this problem would arise during testing, a pilot study was carried out before the main investigation, incorporating both the traditional methodology and the modified one described in section 2.3.1. In the pilot, 11 bees from each of two colonies (22 bees in total) were subjected to a single forward learning task. Each bee was recorded using digital video, but its choice sequence was also scored in real time by an observer. The observer noted the first probing of a rewarding flower, as well as the next 100 choices, which were used to create 11 data points and fit a learning curve for each bee. Learning curves were generated for the same bees through review of the video files, using the first landing on a rewarding flower and the next 100 choices as the basis for data points. Thus, each bee’s learning performance was modeled by two curve equations based on the two different observational methods, the decay constants of which were compared using paired t-tests for all 22 bees tested. The constants did not differ significantly (t = 0.444, df = 21, p = 0.66), so the protocol used in this investigation can be considered comparable with the observational standards established in previous studies (e.g. Raine & Chittka 2008, Ings et al. 2009, Raine & Chittka 2012, Evans et al. 2017).
2.2.9 Statistical analyses:

All statistical calculations were performed using the Stats package in R version 2.15.3 (R Development Core Team 2012). Since worker bumblebees within a colony are all siblings and the colonies themselves are the reproductive units, mean colony LPI was considered as a single replicate for statistical analyses of variation in learning performance within and between cognitive tasks. As such, experiments on different workers from the same colony were considered repeated measurements taken from the same subject, necessitating the use of a repeated-measures design with fewer degrees of freedom. A two-way repeated-measures ANOVA, with task as a within-subject effect and colony as a between subject effect, was used to determine whether the LPI values for each colony differed significantly among tasks. Additional two-way ANOVAs were also applied to LPI values for individual bees within each of the four tasks, to determine whether the variation in LPI within colonies was greater than the variation between colonies. Pairwise comparisons of mean task LPI values were carried out using Tukey’s post-hoc tests.

2.3 Results

2.3.1 Captive rearing of wild B. impatiens colonies:

Of the 154 wild queens that were collected, 89 (57.8%) produced some form of brood (eggs, larvae, or adults), 14 (9.1%) produced adult workers, but only two (1.2%) produced mature colonies that were useable in behavioural experiments (Table 2.1). All but one of the queens that produced adult workers were collected
before May 24. Due to time constraints, parasite screening of fecal samples for *Crithidia bombi, Nosema bombi, Nosema ceranae,* and *Sphaerularia bombi* could not be applied to all of the wild queens, but it was applied to the foundress queens of the mature colonies used in learning experiments. Fecal samples were collected from these queens and from an additional worker from each of their colonies by capturing them in collection vials and monitoring them until they defecated. Each fecal sample, along with a small volume of water, was pipetted onto a slide and examined under a compound microscope. There were no visible flagellate *C. bombi* forms, spores of *N. bombi* and *N. ceranae,* or *S. bombi* eggs or adults within the fecal samples, so the colonies were either uninfected, or infection was not substantial or widespread. Thus, parasitism was not considered to be a factor in the observed patterns of learning performance in this investigation. It is not possible to know what role parasitism played in the failure of the larger population of queens to initiate colonies.

### 2.3.2 Learning performance:

Colony mean LPI scores differed significantly among the four different learning tasks (df = 3, F = 355.1, p < 0.001) for both of the experimental wild colonies. Post-hoc comparison tests revealed that every pairwise comparison of colony LPI scores for two different tasks returned a significant difference (Fig 2.1), even the two forward learning tasks that involved the same rewarded colour and only differed in terms of the bees’ prior experience. The variation in LPI scores within colonies was greater than that between colonies for all learning tasks (Two-way ANOVA: forward task 1: df = 1, F = 0.537, p = 0.47; reverse task: df = 1, F =
0.002, p = 0.97; forward task 2: df = 1, F = 0.059, p = 0.81' novelty task: df = 1, F = 0.059, see Fig. 2.1). Likewise, the innate preference of individuals for blue flowers, represented by the proportion of blue flower choices made before first landing on a yellow flower, was more variable between individuals in a colony than between colonies (df = 1, F = 0.001, p = 0.974). The lowest, or “best” learning performance values were observed in the reversal learning task (mean LPI = 8.515), where bees not only learned faster than in other tasks, but also achieved superior saturation performance levels. The highest, or “worst”, LPI values occurred in the novel colour learning task (mean LPI = 20.69), indicating that bees maintained a high (>0.5) proportion of errors throughout the choice sequence, and thus did not prioritize visits to highly rewarding flowers upon discovering them in the arena.

2.4 Discussion:

Only 9.1% of the wild B. impatiens queens formed colonies from which workers eclosed, an unusually low success rate by the standards of bumblebee captive rearing. Other studies using similar methods have achieved success rates in excess of 30% for North American Bombus species such as B. appositus, B. bifarius, B. terricola, and B. rufocinctus (Plowright & Jay 1966, Strange 2010). Considering that B. impatiens is a readily available commercial pollinator, it could be as amenable to captive rearing techniques as these species. However, the lack of published work pertaining specifically to nesting rates in B. impatiens means that there is no well-established benchmark against which to compare the results of this investigation. Queens that were collected in May produced more viable brood than those collected in June or later (Table 2.1), which appears to reflect the timing of
the species’ life cycle in Southern Ontario, where most wild colonies are founded in the late spring and early summer (Colla & Dumesh 2010).

Bees performing differently in different learning tasks is something that can be reasonably expected, considering that reversal of the rewarding and unRewarding flower colours and the addition of a third colour pose different levels of challenge to foraging bees. The observed pattern of variation in performance across the four tasks allows for further inferences to be made about the cognitive factors that facilitate or constrain learning in *B. impatiens*. First, the consistently better performance of workers in the reversal learning task than in the forward learning tasks is a notable result, as the only difference between these tasks was the rewarding flower colour, as well as the fact that in one task learning was opposed to an innate preference while in the other it was opposed to a recent learned association. Based on the results it appears that wild *B. impatiens* are better at learning and remembering the colour blue than the other two colours used in the learning tasks, with the most likely explanation being the innate preference for blue flowers exhibited by this species. It is possible that there was some additive interaction between this preference and the normal associative learning process, resulting in bees visiting blue flowers with greater frequency and fidelity than they did the rewarding yellow flowers in the previous task. Alternatively, these results could reflect bees simply returning to their innate preference upon discovering that blue flowers are rewarding, but this would still constitute learning as the determination to switch back to blue (and abandon yellow) is still made based on environmental information.
The fact that workers performed significantly worse in the second forward learning task than they did in the first is another conspicuous result, as the physical parameters of both tasks were identical, and suggests some degree of cognitive interference following the double reversal of the colour-reward association. Finally, the LPI scores in the novel colour learning task were extremely poor compared to all of the other tasks, suggesting that workers did not favour the higher reward levels within their foraging bouts. It is unclear if they were unable to achieve this or whether they were not sufficiently motivated to explore alternatives to the yellow flowers that continued to be rewarding. Whilst bees might not have perceived green flowers as clearly as blue and yellow flowers against the green floor of the flight arena, the fact that they regularly approached and landed on green flowers during the task suggest they were aware of their presence and functional similarity to the other flower colours.

As the cognitive experiments conducted in this chapter represent the first application of the visual learning paradigm outlined in Raine et al. (2006b) to wild *B. impatiens*, there is no available point of reference for comparing the learning performance of these wild colonies with that of colonies from other wild populations of the species. However, it is possible to compare their learning performance with that of commercial colonies that have been subjected to the same experimental protocol, as can be seen in Chapter 3.
Figure 2.1: Box and whisker plots representing intra-colony variation in Learning Performance Index (LPI) scores of 15 workers from each of two wild *B. impatiens* colonies in four serial colour learning tasks. The rewarding flower colour varied among tasks: yellow in forward (1 and 2) and blue in reverse tasks. In the novelty task, the newly introduced green flowers were the most rewarding. When bees chose flowers of other colours in these tasks, these choices were categorized as errors. The Learning Performance Index (LPI) represents the number of errors made by bees in each task – hence low LPI scores indicate bees choosing the (most) rewarding flower colour more often. Data from each wild *B. impatiens* colony are shown in different colours: white and grey. In each box the thick horizontal bar is the colony median, whilst the lower and upper edges represent the 25% and 75% quartiles respectively. Whiskers indicate the maximum and minimum values that are not outliers, and outliers are represented by open circles.
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Table 2.1: Summary of the colony-founding activity of all wild *B. impatiens* queens captured from the field for colony rearing. Nest-searching queens were taken from either the University of Guelph campus or the Arboretum. For each queen, the date of capture has been noted, as well as the presence or absence of wax structures within the nest container and the date upon which the first adult workers emerged (if applicable). Queens that produced mature colonies (102 and 117) are shown in bold face.
Chapter 3: Visual learning performance of commercially reared

*Bombus impatiens* colonies

Abstract

Commercially reared bumblebees are economically important as greenhouse pollinators, and are frequently used in studies of insect behaviour due to their availability and tractability in laboratory environments. However, as these bees have been bred in captivity for successive generations, it becomes increasingly likely that they have genetically, morphologically, and behaviourally diverged from their wild counterparts. The objective of this study was to test workers from multiple commercial *Bombus impatiens* colonies in four serial colour-learning tasks, and to compare their performance with that of bees from wild colonies performing the same tasks described in Chapter 2. The first task determined how quickly bees learned to associate the colour yellow with floral rewards, overcoming their innate preference for blue; the following two tasks presented sequential reversals of this colour-reward association; the final task involved learning to associate a novel, green-coloured flower with the highest rewards within an array that also included the original yellow and blue flowers. Workers (*n = 142*) from ten colonies were assessed using these methods. They all displayed the same pattern of inter-task variation in learning performance that was also observed in bees from wild colonies: bees learned more quickly and achieved superior saturation performance when blue was the rewarded flower colour, learned more slowly and achieved inferior saturation performance in the second rewarded-yellow task than in the first, and did not learn to prefer the novel, highly-
rewarding flower colour. There was significant inter-colony variation in learning performance within individual tasks, with the exception of the novel colour learning task. However, there was no evidence that the within-task learning performance of wild colonies was drawn from a different distribution than that of commercial colonies. There is as yet no evidence that domestication has altered the cognitive abilities of *B. impatiens*. However, there is strong evidence that both wild and commercial *B. impatiens* exhibit innate preference for blue flowers over yellow flowers while foraging, experience cognitive interference as a result of serial learning task reversals, and respond to two rewarding flower colours in the same learning task by generalizing their approach and landing behaviour to both rewarded stimuli.

### 3.1 Introduction:

Domesticated bumblebees have been available to growers and researchers for nearly three decades (Velthuis & van Doorn 2006), but it is still unclear what effect mass-breeding and anthropogenic movement has had on these organisms, especially in terms of their cognition and behaviour. It has been reported that repeated rearing of captive *B. terrestris* colonies inadvertently selects for lighter queens (Beekman *et al.* 2000) and may also select for higher mating rates and greater production of reproductive individuals (Ings *et al.* 2006, Ings *et al.* 2009, Inoue *et al.* 2012). Even if there is no intentional artificial selection on the part of commercial suppliers, domesticated colonies may differ from wild ones in terms of their physical and behavioural traits. Also, without replenishment of domesticated stocks with *B. impatiens* queens from the field, colonies reared from these stocks
will experience greater risk of inbreeding depression. An additional factor distinguishing commercial *B. impatiens* populations from wild ones is their apparent greater infection rate by pathogens such as the trypanosome *Crithidia bombi* (Otterstatter *et al.* 2008, Graystock *et al.* 2014), which has been shown to affect foraging behaviour and learning ability in infected individuals (Otterstatter *et al.* 2005, Gegear *et al.* 2005, 2006). Given these considerations, it is entirely possible that the cognitive abilities of commercially reared *B. impatiens* are not reflective of what the species is capable of in the wild.

The objective of this chapter is to assess the learning ability of worker bees from commercial *B. impatiens* colonies following the same methods used on bees from wild colonies (see Chapter 2), and to examine potential differences in cognitive performance between the two populations. A secondary objective is to evaluate how quickly workers from commercial colonies find the novel flower colour in the final, three-colour learning task, and whether this speed of discovery is associated with their performance in earlier tasks. One hypothesis put forward to explain intra-colony variation in learning performance of bumblebees states that individuals prone to foraging errors are quicker to discover new rewarding flower colours, thus granting an unorthodox foraging advantage to poorer learners. So far evidence of this phenomenon has only been reported from commercial colonies of *B. terrestris* (Evans & Raine 2014b), but the present investigation presents an opportunity to evaluate the hypothesis for *B. impatiens* as well.
3.2 Materials and methods:

3.2.1 Procurement of commercial *B. impatiens* colonies:

Ten commercial colonies of *B. impatiens* were acquired from Biobest Canada Ltd. (Leamington, ON) for learning experiments in the laboratory. Logistical constraints made it impossible to conduct experiments on all colonies simultaneously, so they were assessed in four cohorts: cohort 1 (3 colonies) were tested between 14 November and 4 December 2016; cohort 2 (3 colonies) were tested between 7 and 20 December 2016; cohort 3 (2 colonies) were tested between 20 May and 6 June 2017; and cohort 4 (2 colonies) were tested between 7 and 16 August 2017. Although the precise age of the colonies was unknown, on arrival each contained 30-50 workers, one queen, and no additional reproductive individuals, so they were clearly relatively young and showed no signs of senescence during testing. Within 72 hours of arrival, all colonies were transferred to bipartite wooden nest boxes identical to the boxes that wild colonies were reared in (see Chapter 1) and stored in the laboratory. Each colony was fed defrosted honeybee-collected pollen (Overton Apiary, Caledon, ON) and unscented 30% sucrose solution *ad libitum*, but feeding only took place after each day’s experiments were completed to keep satiation from suppressing recruitment of foragers to flight arenas.

3.2.2 Learning experiments with bees from commercial colonies:

The cognitive abilities of worker bees from commercial colonies were assessed using the same four serial colour learning tasks that were administered
to bees from wild colonies (see Chapter 2). Individual bees were pre-trained on four bicoloured artificial flowers rewarded with unscented 30% sucrose solution, and motivated foragers completing five or more bouts in this phase were tagged and used in learning tasks. The aim was to test the learning performance of 15 bees per colony, but this was only possible for seven colonies (for two colonies I tested 14 bees, and for one 13 bees were tested due to bees failing to complete the sequence of tasks within the time frame of recording). During learning tasks, bees were recorded with four Sony digital camcorders (two CX220e models and two CX405 models) using the same protocol outlined in section 2.2.1.

Learning tasks were presented to commercially-reared foragers in the same order that they were presented to wild foragers:

1) A forward learning task with 10 rewarding yellow flowers and 10 empty blue flowers (see section 2.2.3).

2) A reversal learning task with 10 rewarding blue flowers and 10 empty yellow flowers (see section 2.2.4)

3) A second forward learning task with 10 rewarding yellow flowers and 10 empty blue flowers (see section 2.2.5)

4) A novel colour learning task with 10 empty blue flowers, 10 rewarding yellow flowers, and 10 highly-rewarding green flowers (see section 2.2.6).

Video recordings were reviewed to determine the sequence of choices made by each bee before it first landed on a rewarding flower, as well as the subsequent sequence of 100 flower choices. These choice sequences were then translated into 11 data points (see section 2.2.7) that were used to fit learning curves in
Microcal Origin Pro 2017, which were used in turn to generate LPI scores for each bee in each task (see sections 2.2.7 and 2.2.8).

It was not possible to test bees from the two colonies in cohort 4 (in August 2017) for the novel colour learning task due to the need to move them to the Arboretum for a foraging experiment before the end of the field season. Thus, there were only eight colony-level observations for that task, as opposed to 10 colony-level observations for the other tasks. The design of flight arenas and flowers, as well as the quantity and concentration of sucrose solution rewards, were identical for all tasks to those stated in Chapter 2. The same is true for the criteria used to determine whether a bee had completed a task.

3.2.3 Statistical analyses:

All statistical analyses of the data collected in this study were performed in R version 2.15.3 using the stats and boot packages. As with the wild colonies, it was necessary to use a repeated measures design that treated each colony as a subject due to the relatedness of nestmates. At the colony level, a two-way repeated measures ANOVA was used to test for significant differences in LPI values between tasks and colonies. Task was considered a within-subject effect, while colony was considered a between-subject effect. All possible pairs of task LPI scores were compared using Tukey’s post-hoc tests.

At the individual level, two-way ANOVAs were conducted on LPI values in each of the four tasks, to determine whether the variation in LPI in a task was greater between colonies or within colonies. In addition to this, to evaluate the hypothesis that error-prone foragers are more likely to discover new resources
outlined in Evans & Raine (2014b), a Spearman’s rank correlation was computed between forager LPI scores in the first task and the number of choices made by the same foragers (n = 137) before first landing on a green flower in the novelty task.

The sample size of colony-level observations for commercially reared *B. impatiens* (n = 8-10 colonies, this study) was much larger than the sample size for wild *B. impatiens* (n = 2 colonies, Chapter 2), making a comparison of learning performance between the two groups using t tests or analysis of variance statistically untenable. Instead, bootstrap resampling was conducted in the R boot package in order to create a distribution of comparisons between subsamples of equal size. Within each task, the bootstrap algorithm was used to create 10000 replications of the difference in mean LPI values between the sample of two wild colonies and a random 2-colony subsample of the 10 commercial colonies (8 in the case of the novel colour learning task). Confidence intervals (95%) were then constructed for these bootstrap distributions.

3.3 Results:

Colony mean LPI values differed significantly across tasks (df = 3, F = 264, p < 0.001), and every post-hoc pairwise comparison between task means returned a significant difference. The variation in LPI between colonies was greater than that within colonies for forward learning task 1 (df = 9, F = 6.716, p < 0.001) and 2 (df = 9, F = 3.921, p < 0.001), but curiously not for the reversal learning task (df = 9, F = 1.332, p = 0.226) or novelty learning task (df = 7, F = 1.193, p = 0.313). The general pattern of performance across all four tasks was the same as observed for bees from wild colonies (See Fig. 3.1): LPI scores were best (lowest) in the
reversal task (Mean LPI = 7.25) and worst (highest) in the novelty task (Mean LPI = 20.59), and the scores for the first forward task (Mean LPI = 10.24) were better (lower) than those for the second (Mean LPI = 13.16). The correlation coefficient between individual LPI in forward task 1 and time taken to discover a novel flower colour in the novelty task by individual workers (n = 137) was non-significant (R = 0.0479, t = 0.5594, df = 136, p = 0.577).

For each learning task, the bootstrap distribution of differences in mean LPI between the two wild colonies and random subsamples of two commercial colonies had a 95% confidence interval that included 0 (See Figs. 3.2, 3.3, 3.4, 3.5). This means that the inter-colony variability in learning performance within these two populations was greater than the variability between the populations themselves, meaning the null hypothesis of no difference in learning performance between wild and commercial *B. impatiens* cannot be rejected. Thus, the investigation did not find evidence for any cognitive effects of commercial domestication of *B. impatiens*.

**3.4 Discussion:**

Commercial colonies of *B. impatiens* differ significantly from each other in terms of their performance in forward visual learning tasks, but not in the reversal learning task where blue flowers were rewarding or in the novel colour learning task with highly rewarding green flowers. This finding of inter-colony discrepancies suggests that there is heritable variation in learning ability at the level of the reproductive units (colonies) of *B. impatiens*, and that the cognitive traits of the species are therefore subject to selection processes. However, under this interpretation of the data, the lack of significant variation in learning performance
within two of the tasks is an unexpected finding that requires further explanation. In the case of the novel colour task, this result can be attributed to the colonies being either unwilling or unable to learn to prefer green flowers, as their LPI scores in the task were unilaterally poor. Unfortunately, the protocols used to measure learning performance in this investigation cannot detect or quantify motivation on the part of the bees, so there is no way of confirming which explanation applies. This is not the case for the reversal learning task, where all colonies displayed better performance than in any other task but did not differ significantly from each other. All of the colonies used in experiments innately preferred blue flowers, so their generally high level of performance in this task could be a result of innate colour bias interacting with learning processes and pushing performance to near-optimal levels.

Error-prone bees were not significantly more likely to discover a novel flower colour than their more efficient counterparts, a result opposite to that reported by Evans & Raine (2014b) for B. terrestris in a similar experiment. This may represent a difference in foraging strategy between the two species, where B. impatiens is more conservative overall and makes fewer ‘exploratory’ visits to new flower types when it is already constant to a rewarding type.

Commercial colonies do not appear to differ from wild ones in terms of their general pattern of learning performance across the four assigned cognitive tasks. They perform better in the reversal learning task than in the forward learning task, show signs of interference in the second forward learning task, and do not display reduction in error rate that would be indicative of learning in the novel colour task. There is no evidence that wild and commercial colonies differ in terms of learning
performance within the same task, although this finding is likely a natural consequence of the extremely conservative method of analysis through bootstrapping necessitated by the large discrepancy in sample sizes. As a result, the data collected here are insufficient to conclude that there are no meaningful cognitive differences between wild and commercial *B. impatiens*. Further research will be necessary to settle this question, with greater numbers of wild colonies, and ideally colonies from different regional populations as well.
Figure 3.1: Box and whisker plots representing colony mean Learning Performance Index (LPI) scores for 10 commercial *B. impatiens* colonies in a serial colour learning task. The rewarding flower colour varied among tasks: yellow in forward (1 and 2) and blue in reverse tasks. In the novelty task, the newly introduced green flowers were the most rewarding. When bees chose flowers of other colours in these tasks, these choices were categorized as errors. The Learning Performance Index (LPI) represents the number of errors made by bees in each task – hence low LPI scores indicate bees choosing the (most) rewarding flower colour more often. In each box the thick horizontal bar is the colony median, whilst the lower and upper edges represent the 25% and 75% quartiles respectively. Whiskers indicate the maximum and minimum values.
Figure 3.2: Bootstrap distribution of the difference in mean learning performance (LPI) between two wild and two randomly subsampled commercial *B. impatiens* colonies for forward learning task 1 (10,000 random resampling events). The bounds of the 95% confidence interval are indicated by vertical red lines.
Figure 3.3: Bootstrap distribution of the difference in mean learning performance (LPI) between two wild and two randomly subsampled commercial B. impatiens colonies for the reversal learning task (10000 random resampling events). The bounds of the 95% confidence interval are indicated by vertical red lines.
Figure 3.4: Bootstrap distribution of the difference in mean learning performance (LPI) between two wild and two randomly subsampled commercial *B. impatiens* colonies for forward learning task 2 (10000 random resampling events). The bounds of the 95% confidence interval are indicated by vertical red lines.
Figure 3.5: Bootstrap distribution of the difference in mean learning performance (LPI) between two wild and two randomly subsampled commercial *B. impatiens* colonies for the novel colour learning task (10000 random resampling events). The bounds of the 95% confidence interval are indicated by vertical red lines.
Chapter 4: Field foraging experiment on commercial *Bombus impatiens* colonies previously tested for learning performance

**Abstract**

The visual learning ability of bumblebees has been well-studied in a laboratory context, but relatively little is known about how bees use their cognitive abilities in natural environments, and how this relates to the fitness of their colonies. In laboratory settings, learning performance is associated with the rate of rewarding flower visits made a bee, which can be expected to translate to greater net energy gain during foraging in the field. However, there are also potential drawbacks of improved learning ability that need to be evaluated in the field before conclusions can be drawn regarding the impact of learning on colony fitness. The objective of this chapter was to study the relationship between learning ability and foraging performance for *B. impatiens* workers. First the learning performance of workers (n = 34) was assessed in the laboratory, then their colonies (n = 4) were moved to a field site where they were allowed to forage freely during daylight hours over a 7-day period. Foragers were fitted with passive RFID transponders which recorded the frequency and duration of their foraging bouts, and were weighed every time they left or re-entered their home colonies. The combined weight of pollen and nectar brought back by each forager was measured on both a per-bout basis and as a total value across all completed bouts, and these measurements were then compared with the established learning performance of these foragers. Bees with the best learning scores (making fewer errors)) collected significantly more pollen and nectar per bout than those with higher LPI scores, but did not
collect more resources over the duration experiment, likely due to their lower activity levels outside the colony. This result suggests one means by which different behavioural types may be maintained within a colony: fast learning workers are more efficient foragers during a shorter timeframe, while slower learning workers may provide more foraging output in the long run.

**4.1 Introduction:**

Most of the existing knowledge of how bumblebees learn and remember information comes from laboratory experiments in a simplified foraging environment. These experiments have been useful in elucidating how bees process one or two types of information—such as flower shape, flower colour, olfactory cues, or predation risk—while eliminating or holding constant all others. However, they do not approach the complexity of the cognitive challenges that wild bees face in the field, where all of these types of information are simultaneously available and variable. Field foraging could be considered the most important context for the study of cognition, as this is where selection on cognitive traits is presumably taking place (Chittka et al. 2004), and also where any concerns about the intersection of cognitive traits with bumblebee conservation must be addressed (Gill & Raine 2014).

In order to address the sizeable knowledge gap regarding interaction between bumblebee cognitive traits and the natural floral marketplace, studies that relate existing measures of cognitive ability to field foraging efficiency—and by extension fitness—are required (Thornton et al. 2014). Some early efforts in this direction include Raine & Chittka (2008) and Evans et al. (2017), both of which put
colonies of *B. terrestris* through a simple laboratory learning experiment before allowing them to forage outdoors and quantifying resource collection. The former study focused primarily on nectar collection at the colony level and found colonies containing faster-learning workers were better performers in the field (Raine & Chittka 2008), while the latter examined both pollen and nectar collection at the individual level and found fast learning was actually detrimental to productivity (Evans *et al.* 2017). These seemingly contradictory results demonstrate some of the complexity present in this frontier of cognitive ecology: are the fitness effects of learning ability dependent on local environment to the point that they can be significantly positive or negative at different locations or points in time? Or are certain cognitive traits beneficial at the colony level while deleterious at the individual level? The answer is likely to depend on whether there are individual-level and colony-level costs associated with learning ability, and what these costs entail, both physiologically in terms of lifespan and activity level, and behaviourally in terms of speed and accuracy of decisions. The purpose of this section of my thesis investigation ways to further examine these questions through the use of a similar methodology to those preceding studies. A subsample of commercially-reared colonies tested in Chapter 3 were allowed to forage freely in an outdoor environment, and the activity levels, efficiency, and overall productivity of foragers were measured so that they could be related to learning performance scores compiled in the laboratory. This is the first application of this methodology to *B. impatiens*.
4.2 Materials and methods:

4.2.1 Experimental colonies:

Bees from four commercial *B. impatiens* colonies that underwent cognitive testing in the laboratory (see Chapter 3) were selected for the field foraging experiment. These were divided into two cohorts, each containing two colonies:

1) The learning performance of bees from the first two colonies was assessed in the laboratory between May 20 and June 6 of 2017. Foraging data were collected from these colonies on seven days between June 21 and June 31. Data could not be collected on June 23, 25, 29, and 30 due to inclement weather.

2) The learning performance of bees from the second two colonies was assessed in the laboratory between August 7 and August 16. Foraging data were collected from these colonies on seven days between August 19 and August 27. Data could not be collected on August 20 and 24 due to inclement weather.

Colonies were placed outdoors for a ~5 hour period each day when foraging data were being collected. This foraging period was continuous for all colonies on all data-collecting days, but the start time varied between 8:00 am and 12:30pm due to variation in weather conditions and equipment set-up time. At all other times, including overnight, the bees were sealed inside their nest boxes and stored indoors. On days where bees were not able to forage due to rain, the colonies were provided *ad libitum* with 30% sucrose solution to limit starvation-induced worker mortality. However, colonies were not fed at all on days where they
foraged, so satiation was unlikely to have affected the natural rate of worker recruitment to foraging.

During the experiment, tagged workers from one colony occasionally entered—and were accepted by—the other experimental colony. At the end of the data-collecting day, when the colonies were being stored indoors, these workers were relocated to their colony of origin with long forceps.

4.2.2 Field site:

The field foraging experiment was conducted in the Gosling Wildlife Gardens at the University of Guelph Arboretum. Both pairs of colonies were placed in the same location within the gardens (The “Small City Garden,” coordinates 43°32’31.0”N 80°12’40.3”W) for foraging data collection, although they were present at different points in time during the summer. This site contains a wide variety of plants offering flowers ranging widely in colour and morphology, not all of which are attractive to or accessible by bumblebees. The five different garden sections are also separated by hedgerows that added complexity to the foraging environment. Based on these features, the Wildlife Gardens were deemed suitable for an experiment relating learning ability and field foraging efficiency because they contained ample floral resources for supporting two bumblebee colonies without those resources being so readily available as to provide no cognitive challenge.

4.2.3 Tracking Forager Activity

For each colony used in the field experiment, all individual workers that underwent learning tests in the laboratory were fitted with Radio Frequency
Identification (RFID) tags (Microsensys® mic3-Tag 64 bit read only transponder; carrier frequency: 13.56 MHz, dimensions: 1.9 x 1.6 x 0.5mm; mass: 4mg), which were glued on top of the Opalith tags that the bees had previously been fitted with during the laboratory learning experiments. The identifier number for each RFID tag was noted before that tag was affixed to a worker, so that the worker’s identity could be tracked moving forward from the laboratory trials.

Before each day of field data collection, two RFID reader modules (Microsensys® iID MAJA reader module 4.1) were positioned at each nest box entrance such that bees needed to pass through both modules in order to enter or exit their colony. These were connected to an autonomous data-logging device (Microsensys® iID Host type MAJA 4.1) that recorded every instance of an RFID-tagged worker passing through one of the reader modules. In other words, it recorded the total number of foraging bouts completed by tagged workers, as well as the length and timing of each individual bout.

4.2.4 Measuring forager productivity:

In addition to the RFID reader modules, an electronic balance (Sartorius Practum 213-1S) was placed at the entrance of each nest box so that the weight of departing and returning workers could be recorded as a measurement of foraging productivity. The weighing platform was positioned between the two modules, which were connected by a transparent Perspex tunnel. The Perspex floor was removed from middle section of the tunnel, forcing bees to walk directly over the weighing platform on their way in and out of the colony. Access to this section was restricted on both ends by gates that could either be in an “open” position, which
allowed bees to pass through one at a time, or a “closed” position, that did not allow any bees to pass through. These gates were used to prevent multiple bees from stepping on the weighing platform at once. They also slowed the progress of bees, preventing them from flying partially or entirely over the weighing platform and producing inaccurate mass measurements. Since these measurements were being taken continuously and simultaneously from two colonies, the displays from both balances were recorded using Sony CX405 camcorders to ensure that no readings were missed. The cameras and RFID data loggers were activated and deactivated at the same time each day, so the timestamps from both recording devices were synchronized. It was therefore possible to associate weight readings with specific tagged workers by examining the same timestamp on both devices.

4.2.5 Statistical analyses:

For each tagged worker, the number of days spent foraging and the number of bouts per day were determined using the RFID reader data. The video footage of the electronic balance readout was then used to determine the departing and returning weight for each foraging bout, and the difference in these weights was taken to represent the amount of food resources (pollen+nectar) brought back to the colony. This information was then simplified into two measures of productivity for each worker: total pollen and nectar collection across all bouts, and pollen and nectar collection per bout. Since several workers did not return to their colonies near the end of the data-collection period and thus could not be measured or weighed at the experiment’s conclusion, the average departing mass of each
worker across all of its foraging bouts was used as the measure of worker size in the analysis.

In order to assess the relationship between learning performance and field foraging activity and productivity while accounting for environmental and colony-level effects general linear mixed-effects models were constructed using the nlme package in R version 2.15.3. Four different models were fitted for four response variables: 1) mean nectar and pollen collection per bout, 2) total nectar and pollen collection, 3) number of days spent foraging, and 4) mean foraging bouts per day. Each model was constructed using the same process of stepwise parameter addition, and the Akaike Information Criterion (AIC) was used as the basis for model selection. First, a basic model that included only colony and cohort as random effects was fitted, then worker mass and colony age were added in stepwise fashion as fixed effects. From these models, the one with the lowest AIC value was selected, and LPI in Forward learning task 1 (see Chapter 3) was added to it as a fixed effect. If this added parameter significantly lowered the model AIC, then it was considered to be predicting change in the response variable.

4.3 Results:

A total of 34 workers across all colonies completed at least one foraging bout during the field experiment. When foraging productivity, either over all seven days of foraging or on a per-bout basis, was the response variable, the best model prior to the addition of LPI as a fixed effect was the simplest one, with only colony and cohort included as random effects. The addition of LPI as a predictor improved
the AIC value for the mean pollen and nectar collection per bout model, but not for the total pollen and nectar collection model.

For models where the dependent variable was forager activity, measured either in days spent foraging or mean bouts per day, worker mass improved AIC values while LPI did not notably affect them (Table 4.1).

Even accounting for the random effects of colony and cohort identity, there was a significant negative correlation between LPI and mean pollen and nectar collection per bout (model coefficient = -0.00516±0.00109, t = -4.732112, p < 0.001, Fig. 4.1). This means that bees which committed fewer errors and learned faster during the laboratory testing period were more efficient foragers on a per bout basis than their slower learning counterparts. However, LPI was not significantly correlated with any of the other response variables used in the analysis (Figs. 4.2, 4.3, 4.4).

4.4 Discussion:

Bees that demonstrated fast learning in the laboratory were more efficient as foragers, but did not contribute more resources to the colony over their lifetime than their slower learning nestmates and conspecifics. This result may be explained in part by a negative effect of learning ability on activity level, a phenomenon which has been observed in B. terrestris workers and has been proposed to represent a potential physiological trade-off with greater investment in neurological tissue associated with learning ability (Evans et al. 2017). However, no significant effects on measures of activity were detected in this experiment, though it must be noted that the relationship between these two traits could not be
examined in detail due to the comparatively large effect of worker size on activity (Table 4.1). An experiment with a larger sample size of either similarly sized workers or clearly-defined size cohorts would be better suited to elucidating this relationship. In a similar study with *B. terrestris* colonies, Raine & Chittka 2008) found that colonies with faster-learning workers had higher rates of nectar foraging per unit time, which seemingly agrees with the conclusion of this experiment that learning performance is positively linked with foraging efficiency. However, that study only examined foraging at the colony level, and did not track the performance of individual foragers of known learning ability. On the other hand, Evans *et al.* (2017) found that learning performance was not linked with foraging efficiency in individual *B. terrestris* workers, but had an apparent negative effect on the amount of time spent foraging. In that study, though, colony level effects of learning performance were not examined and foraging efficiency was measured differently than in the present experiment. Judging from this previous research as well as the results presented here, it appears that workers with superior learning ability can improve the foraging efficiency of their colonies, but perhaps only for a limited time as they may be less active foragers than other workers. The fact that that workers with higher learning performance scores were more efficient but not more productive overall suggests that the fitness effects of learning ability are complex, and their directionality is likely dependent on environmental factors. These factors cannot be controlled within a field setting, but future studies using this methodology might shed some lights on their effects by using multiple field sites, characterized using a combination of Geographical Information Systems (GIS) and quantitative methods such as ordination. If the physical environment is in fact the most
important determinant of the value of learning ability, and certain environmental traits are strongly associated with cognitive challenges, then one would expect to see variation in the productivity of high-level learners along the axes that represent these traits.

Figure 4.1: Relationship between Learning Performance and mean pollen and nectar collection per foraging bout in *B. impatiens* workers. Bees making fewer errors (lower LPI) had a significantly higher foraging efficiency. Circles, squares, triangles and crosses indicate bees tested from each of the four different colonies used in the experiment.
Figure 4.2: Relationship between Learning Performance and total pollen and nectar collection in *B. impatiens* workers. Circles, squares, triangles and crosses indicate bees tested from each of the four different colonies used in the experiment.

Figure 4.3: Relationship between Learning Performance and the number of Days spent foraging over a 7-day period in *B. impatiens* workers. Circles, squares, triangles and crosses indicate bees tested from each of the four different colonies used in the experiment.
Figure 4.4: Relationship between Learning Performance and mean foraging bouts per day in *B. impatiens* workers. Circles, squares, triangles and crosses indicate bees tested from each of the four different colonies used in the experiment.

<table>
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<th>Response Variable</th>
<th>Mean Foraging Bouts Per Day</th>
<th>Total Nectar+Pollen</th>
<th>Days Spent Foraging</th>
<th>Mean Bouts/Day</th>
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<td>Mean Nectar+Pollen/Bout</td>
<td>Total Nectar+Pollen</td>
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</table>

Table 4.1: Akaike Information Criterion (AIC) values for candidate models predicting forager efficiency, productivity, and activity. AIC values that were significantly reduced by the addition of a parameter are highlighted in bold face.
Chapter 5: Discussion and conclusions

5.1: Visual learning performance of wild and commercial *Bombus impatiens*

Wild and commercial colonies displayed consistent patterns of learning performance across the four visual learning tasks. For all colonies, the lowest LPI scores (fewest errors) were recorded during the first reversal learning task (during which blue flowers were rewarding), the highest LPI scores were recorded for the novel colour learning task, and scores became significantly worse between the first and second forward learning tasks. The drop off in performance between forward learning tasks suggests that reversal learning causes cognitive interference, but it is interesting that this only manifests itself after two reversals, as bees actually learned the blue-reward association faster and with higher fidelity even after being trained on the yellow associated reward. In previous studies, bumblebees and honeybees have exhibited signs of interference and increasingly generalized responses to two stimuli following serial reversals of their rewarding status (Mota & Giurfa 2010, Strang & Sherry 2014), so the increase in LPI scores in the second learning task is not an entirely unexpected result. On the other hand, the ability to quickly process and adjust to a shift of rewards from yellow to blue flowers, which has also been displayed by *Bombus terrestris* (Raine & Chittka 2012), may represent a unique quality of these species and thus merits further examination. There is no inherent behavioural explanation for this capacity, so it is more likely to be a consequence of the neurophysiological evolution of *B. impatiens* and *B. terrestris* in particular. Many species of bumblebee, including *B. impatiens*, display an innate preference for blue flowers, with naïve individuals seeking out these flowers over
all others during their first foraging bouts (Lunau et al. 1996, Raine et al. 2006a, Forrest & Thomson 2009). The bumblebee visual system is particularly sensitive to blue, ultraviolet, and green wavelengths, with less resolution in yellow and red (Briscoe & Chittka 2001), so this may simply be a matter of bees beginning the foraging process with the flowers that are most conspicuous to them (Forrest & Thomson 2009, Rivest et al. 2017). However, the fact that strong blue preference is not universal across bumblebee populations (Gegear & Burns 2007, Ings et al. 2009) suggests that innate colour preference may also be a product of selection for attraction to the floral phenotypes that are most rewarding in a given environment (Raine & Chittka 2007).

Whatever the underlying cause, the observation of improved performance in the reversal learning task is most likely a case of innate and learned behaviours reinforcing each other.

The universally poor LPI scores observed in the novel colour learning task suggest that bees did not develop any learned preference for highly rewarding green flowers during the experiment. Both *B. impatiens* and *B. terrestris* are easily capable of judging small differences in sugar reward concentration (Konzmann & Lunau 2014, Muth et al. 2015a), so the apparent absence of discrimination in this experiment is a curious result. It is possible that bees perceived the difference in reward level but did not learn to associate each level with the appropriate colour. It would be strange for them to be incapable of this (or to ignore this pertinent information), as *B. impatiens* has successfully learned multiple colour-reward associations when foraging for different resources (Muth et al. 2015b), but it is possible that this capacity does not extend to different quality levels of the same resource. A more probable explanation is that the
high baseline reward concentration used in the laboratory trials acted as a disincentive for subsequent learning. With the number of rewarding flowers effectively doubled from the second forward learning task to the novel-colour learning task, and both reward levels greatly exceeding ecologically realistic concentrations, the bees likely adopted a fast but imprecise foraging strategy to visit as many rewarding flowers as possible in as little time as possible (Burns & Dyer 2008). It would be worthwhile to determine whether this strategy changes depending on environmental conditions by repeating the novel colour learning task with considerably lower reward concentrations or with a larger discrepancy in reward levels.

Bee behaviour after encountering the highly-rewarding green flowers may be explained by a generalization response where different reward levels are treated the same in order to facilitate faster but less precise foraging. There are also questions raised by their behaviour before encountering these flowers, which differs markedly from observations made on *B. terrestris* by Evans & Raine (2014b). In that study, which also put workers through a forward learning task with rewarding yellow flowers followed by a task with 10 additional highly-rewarding green flowers, found bees that were more error-prone in the first task were quicker to discover green flowers in the second. This phenomenon could facilitate resource exploration by the colony, and was proposed as a possible explanation for the persistence of poor learners among the foraging contingents of bumblebee colonies (Evans & Raine 2014b). The similarity of the protocols allowed for this hypothesis to be evaluated in the present investigation, but in this case no significant relationship could be detected between LPI scores and the number of flower choices made before landing on a green flower. One might expect that
error-prone bees would be less flower constant at their saturation performance level, and more likely to make an “erroneous” flower choice at any given moment, but this is not what was observed for *B. impatiens* in the novel colour learning task. Perhaps some number of the initial visits to green flowers in this task represented “intentional” exploratory behaviour on the part of the bees, but the protocol contains no means of distinguishing such exploratory visits from “errors.”

No significant difference could be detected between the wild and commercial *B. impatiens* colonies tested in terms of Mean LPI scores within any particular learning task, so there is as yet no evidence of cognitive divergence resulting from domestication in bumblebees. However, there was little statistical power supporting this result due to the small number of wild colonies featured in this investigation and the conservative nature of the bootstrap resampling procedure used to compare populations. While the possibility of interpopulation differences in learning ability should not be completely discounted (Chittka *et al.* 2004, Ings *et al.* 2009), the finding that all colonies followed the same pattern of relative performance across tasks implies that if these differences exist, they are not drastic enough to affect the fundamental sensory and cognitive properties of *B. impatiens*.

### 5.2: Inter-colony and intra-colony variation in visual learning ability

Although variation in learning ability as defined by LPI was observed within both the wild and commercial colonies, colony identity was ultimately the most important source of variation in this investigation. This finding is consistent with several previous studies using multiple colonies of *B. terrestris* in the same type of visual learning
paradigm (Raine et al. 2006, Ings et al. 2009, Raine & Chittka 2012). Although there are also studies that did not find significant variation at the colony level (Evans & Raine 2014a, 2014b), quantifying this type of variation was not their primary objective and their sample sizes were smaller than five colonies, so those particular findings may be statistical artifacts. As colonies are the unit of reproduction in bumblebees, this inter-colony variability represents the most straightforward scenario one would expect to see assuming that learning performance is a heritable trait under selection. In this scenario, individual worker performance is only relevant insofar as it contributes to colony nutritional intake and, by extension, the number and quality of reproductive individuals produced at the end of the colony cycle (Raine & Chittka 2008). As B. impatiens workers within a colony are generally full siblings (Payne et al. 2003), there is limited room for genetic variability within colonies as opposed to between colonies. Intra-colony variation is present, but only within the greater context of some colony-wide level of aptitude: in environments in which learning ability is favoured, one can expect selection to result in higher learning scores for all bees within the colony, raising both the effective “ceiling” and “floor” for these scores.

While the heritable component of variation in cognitive ability occurs at the colony level, the fact that intra-colony variation exists at all is still a phenomenon requiring explanation. Morphological and behavioural heterogeneity is a feature of many insect societies, but has traditionally been considered only in the context of castes and division of labour, where there are clear adaptive explanations for observed differences between individuals (Jandt et al. 2014). The most common example of this is reproductive division of labour, a feature of all eusocial species wherein only certain castes develop
reproductive faculties and are assisted in raising offspring by sterile workers. Intra-colony differentiation of individuals is more elaborate in the highly-developed social systems of ants and termites that can have multiple distinct worker and soldier castes that differ vastly in their size and behavioural routines (Wilson 1976, Noirot 1985). The “castes” and behavioural syndromes of bumblebees are not as pronounced as this, but are present nonetheless: there can be nearly tenfold variation in worker body size within a single bumblebee colony (Mares et al. 2005), and while different sized workers are not differentiated by any specialized morphology, the larger ones are much more likely to become foragers than their smaller counterparts, which spend most of their time performing internal maintenance on the colony (Goulson 2002). Reproductive castes can have adaptive value under conditions of kin selection (Hamilton 1972), while soldiers or defensively-oriented workers can confer enough intrinsic fitness benefit to their colonies to outweigh their lost potential contributions to reproductive output (Roux & Korb 2004). In the case of learning ability, however, it is not at all obvious why foragers should display differing levels of proficiency. In environments where learning is at all useful, one would expect the fastest, most precise learners to acquire more food and contribute more to colony fitness than their slower, less precise counterparts, yet these poor learners are still a fixture within each colony’s contingent of foragers.

Several theories have been proposed to explain the persistence of slow-learning individuals within colonies. The most straightforward of these simply ascribes cognitive differences to size- and experience-dependent brain plasticity. There is indeed some evidence that points towards this type of phenomenon, at least in the physical dimension: body and brain size can vary up to tenfold within a single bumblebee colony.
and the size and density of worker mushroom bodies appears to depend on the accumulation of experience while foraging (Goulson et al. 2002, Mares et al. 2005, Riveros & Gronenberg 2010, Jones et al. 2013, Li et al. 2017). Behavioural studies incorporating size and experience as covariates with learning ability have to this point yielded mixed results: no consistent intra-colony effects of worker age have been found (Raine et al. 2006b, Riveros & Gronenberg 2009, Evans & Raine 2014a), while body size effects have been found within some learning assessment protocols (Worden et al. 2005), but not others (Evans & Raine 2014b). The design of experiments in the present investigation precluded the possibility of closely examining the effects of size and experience, as workers were not evenly distributed among size classes, age was tracked only at the colony level level, and all bees were essentially naïve when they began the learning tasks. It should be noted, however, that size was not taken into consideration for the selection of experimental subjects, a process that was informed solely by recruitment during the pre-training phase. Since smaller individuals are more likely to avoid foraging and preferentially perform tasks within the nest (Goulson et al. 2002), it is likely that the sample of foragers used in this investigation was biased towards the upper end of the worker body size distribution within each colony. While this is reflective of how wild colonies allocate foragers under ecologically realistic conditions, experiments explicitly searching for size effects may benefit from taking a sample of the smallest workers for learning experiments, even if these workers rarely leave the colony.

Another possible underlying cause of differential learning ability among nestmates is sublethal effects of pesticides or pathogens. Although they are not as
obvious or well-studied as lethal effects, these represent an issue of particular ecological and economic importance as their causes are linked to the anthropogenic management of domesticated bees and agroecosystems (Otterstatter et al. 2008, Stanley et al. 2015). Neonicotinoid pesticides and common parasites such as *Crithidia bombi* or *Nosema ceranae* have been found to impair learning ability in bumblebees (Gegear et al. 2006, Stanley et al. 2015, Piiroinen & Goulson 2016), and it is possible that individuals from the same colony may face differential exposure to these factors during foraging, resulting in differential levels of impairment. This phenomenon can only be considered as a hypothetical at the moment, as it has not been the subject of empirical study, and can be ruled out as a possible explanation of the intra-colony variation in learning performance observed in the present investigation. With the exception of the field foraging experiment which took place after all learning tasks were completed, there was no opportunity for any of the colonies used in this investigation to be exposed to pesticides. Also, the wild colonies were screened for parasites and showed no signs of infection (see Chapter 2).

The final and perhaps most intriguing possible explanation of intra-colony variation in learning is that it is, in fact, an adaptive trait selected for at the colony level. At first glance, this hypothesis appears to contradict the conventional wisdom regarding the value of cognitive ability. Given the existing evidence that learning is selected for at the colony level, one would expect to see the same directional selection across all individuals within a colony, but this does not appear to be the case. The possibility that colonies can realize fitness benefits by containing a range of cognitive phenotypes must therefore be considered.
Slower, inaccurate learning may not be adaptive in a contextual vacuum, but if there are behavioural trade-offs involved it may in fact be beneficial for certain individuals. For example, bees that evaluate floral cues before feeding may visit flowers at a slower rate than bees that do not take the time to discriminate. This compromise between speed and accuracy will harm poor learners in situations where errors are costly, but may benefit them if different types of flowers are similarly rewarding and easily accessible (Chittka et al. 2003, Burns & Dyer 2008, Chittka et al. 2009). Another potential trade-off exists between learning and memory-related problem-solving, as bees that are quick to learn and remember a given association may have more difficulty adjusting to reversals of that association, or acquiring entirely new associations. So far, empirical testing of these theories has yielded promising but incomplete results: speed-accuracy trade-offs have been observed in the colour learning and traplining behaviour of bumblebees during simulated foraging bouts (Chittka et al. 2003, Ohashi & Thomson 2013), but this does not extend to impairment of learning reversed associations (Raine & Chittka 2012). Likewise, error-prone bees have been found to be quicker at discovering new rewarding flower types than their more discriminating nestmates in a visual learning paradigm similar to this investigation’s novel colour learning task (Evans & Raine 2014b). In the present investigation, bumblebee learning performance was worsened by multiple reversals of the colour-reward association, but there was no correlation between LPI values for any pair of tasks, and therefore no evidence that faster learners experienced greater levels of interference than slower learners. There was also no relationship between initial LPI values and the speed at which bees discovered a highly-rewarding flower colour, so there is no evidence that intra-colony
cognitive variation plays a role in resource exploration by *B. impatiens*. Based on these findings it appears that there are no intrinsic benefits to slower learning or more erratic foraging behaviour in this species, or that if there are any such benefits, they are not detectable within this visual learning paradigm. It is, however, possible that the major trade-offs associated with learning in this species are physiological, a hypothesis that was evaluated to a certain extent in the field foraging experiment.

5.3: Relationships between learning and foraging performance in the field

Within the four colonies that were used in the field foraging experiment, workers with superior learning ability collected more food on a per-bout basis, but did not differ from their slower-learning counterparts in terms of overall food collection over the duration of the experiment. This result suggests that there may indeed be adaptive value to different cognitive phenotypes within a colony foraging under ecologically realistic conditions: the “smartest” workers are the most efficient foragers, but are either less active or not as long-lived as their slower-learning counterparts that can contribute as much or even more to the colony by simply completing more foraging bouts. To date there has only been one other study of the relationship between visual learning and foraging efficiency in individual bumblebees, which similarly concluded that faster learners did not collect more food resources than slower learners over their lifetime (Evans *et al.* 2017). The causes of this discrepancy in foraging performance are currently unknown, but physiological trade-offs associated with neurological development are the most likely explanation. Learning in bumblebees is associated with investment in the mushroom bodies (Jones *et al.* 2013, Li *et al.* 2017), which could have
sufficient energetic and genetic costs to reduce the activity levels or even the lifespans of individual workers. This particular trade-off was not directly observable by the methodology of the field foraging experiment but can be inferred from the observed data patterns, and would likely have become more obvious had the colonies been allowed to forage for longer than a week. The sliding scale of learning ability in *B. impatiens* thus appears to separate workers into “efficiency” specialists and “volume” specialists during foraging. It is possible for colonies to benefit from both specializations depending on fluctuations in weather conditions, environmental complexity, and resource availability. There is likely to be stabilizing selection maintaining a certain degree of developmental plasticity in the neurological mechanisms underlying learning in bumblebees.

5.4: Conclusions

Using a combination of laboratory learning tests and field experiments, this investigation quantified both the learning ability and field foraging performance of multiple colonies of wild and domesticated *B. impatiens*. The laboratory trials revealed certain cognitive properties of *B. impatiens* that had previously been observed in other bumblebee species, including a strong innate preference for blue flowers and cognitive interference resulting from multiple reversal learning. Bumblebees did not discriminate meaningfully between different levels of nectar reward associated with different colours in this investigation, nor did their cognitive abilities relate to their discovery rate of novel flower colours. The observation of inter-colony variation conforms to the accepted view that cognitive traits can be the subject of natural selection at the colony level, but the general similarity between wild and commercial colonies suggests that domesticated
bees have not experienced artificial selection or genetic drift resulting in cognitive divergence. In the field, superior learners demonstrated more efficient foraging but did not contribute more overall food to their colonies than other bees. The most probable explanation is some physiological cost of learning that reduces lifetime foraging activity, although this could not be tested directly in this study.

Learning and memory play an important role in the foraging behaviour of flower-visiting insects, and can affect the fitness of both these insects and the plants with which they associate. The present investigation represents an attempt to measure multiple aspects of learning performance in an ecologically and economically relevant model system, while relating these observations to a reliable measure of fitness. The visual learning paradigm employed herein was developed in studies of *B. terrestris*, but this investigation has established that it is readily applicable to both wild and domesticated *B. impatiens*, and has found evidence that these species differ in their resource exploration behaviour. It has also established the first conclusive link between learning performance and foraging efficiency in *B. impatiens*, while discovering a possible adaptive mechanism for the maintenance of the intra-colony variation in cognitive ability that has been observed in many bee species. Integrating measures of cognition and fitness into a single study in this manner is an important step on the path to understanding how insect cognition has evolved and continues to evolve in the changing floral markets of the world.
References


cues to minimize intrusion by outsiders. *Proceedings of the Royal Society B. Biological Sciences* 282: 20142750


