THE EFFECTS OF β-SITOSTEROL-β-D-GLUCOSIDE (BSSG) AND PARAQUAT ON SUCROSE SENSITIVITY, OLFACTORY CONDITIONING AND MOTOR DEVELOPMENT IN HONEY BEES, *APIS MELLIFERA*.

Joanne Lemmon

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ABSTRACT

The simple nervous system of the honey bee compared with vertebrate species makes it a good animal for studying effects on behaviour and neurology. Development is not complete at adult emergence and bees continue to mature during the first eight to ten days of adult life. The cuticle and body hairs stiffen and gland development and fat body growth is completed within the first five days (Winston, 1987).

Amyotrophic lateral sclerosis-parkinsonism dementia complex (ALS-PDC) is a progressive neurological disease found predominantly on the island of Guam, and it presents with features of Alzheimer's disease, Parkinson's disease and ALS (Stone, 1993). Flour made from cycad seeds has been implicated in the onset of ALS-PDC, and has been found to impair motor, olfactory and cognitive abilities in mice (Wilson et al., 2002; Shaw & Wilson, 2003). A sterol glucoside, β-Sitosterol-β-D-Glucoside (BSSG), was identified as the most likely molecule within the cycad flour to be causing the impairments (Wilson et al., 2002) and is toxic to cells in the rat cerebral cortex (Khabazian et al., 2002).

BSSG was administered to honey bees in food at concentrations of 20 nM, 2 µM and 200 µM. A control group was fed untreated food as a comparison. Bees treated with the herbicide paraquat were also examined because, like BSSG, it targets dopamine pathways of the brain. Paraquat is an herbicide that is toxic to many species and leads to a dose-dependent reduction in dopaminergic neurons and a decrease in locomotor activity in mice (Brooks et al., 1999). Effects of BSSG and paraquat on honey bee locomotor activity were examined using the following

assays: motor activity, ability to right after being flipped, and performance in a climbing test. Motor abilities were tested in adult worker bees at ages of one, four and six days after emergence. To examine potential effects of BSSG and paraquat on sensory processing, sucrose sensitivity was measured in seven-day old bees by presenting them with solutions of 0.1%, 3%, 10%, 30% and 55% sucrose. Learning ability was tested in eight-day old bees using single trial olfactory conditioning with eugenol scent as the conditioned stimulus; either 10% or 55% sucrose solution was used as the unconditioned stimulus.

BSSG did not affect motor behaviour but paraquat effects were seen in one-day old bees in righting, and in four- and six-day old bees in flying behaviour. BSSG and paraquat both impaired honey bees' responses to sucrose, but neither were found to affect single trial olfactory conditioning. At one week of age worker honey bees also do not respond well to low and moderate sucrose solutions. The results also show that behavioural development continues after honey bees emerge as adults. One-day old bees are unable to fly and some motor behaviours are still developing until bees are four to six days old.

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LIST OF ABBREVIATIONS

ALS – Amyotrophic lateral sclerosis

ALS-PDC – Amyotrophic lateral sclerosis-parkinsonism dementia complex

BP – Brood pheromone

BSSG – β-Sitosterol-β-D-Glucoside

CR – Conditioned response

CS – Conditioned stimulus

MPP^+ – 1-methyl-4-phenylpyridinium

MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

PDC – Parkinsonism-dementia complex

PER – Proboscis extension reflex

QMP – Queen mandibular pheromone

UR – Unconditioned response

US – Unconditioned stimulus

1. INTRODUCTION

1.1 β-Sitosterol-β-D-Glucoside (BSSG)

Amyotrophic lateral sclerosis-parkinsonism dementia complex (ALS-PDC) is a progressive neurological disease predominantly found on the island of Guam (Schulz et al., 2003; Wilson et al., 2003). It has features of Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS), which is also called Lou Gehrig's disease or motor neuron disease (Stone, 1993; Wilson et al., 2004). During the decades following World War II, there was a much higher incidence of neurological disease in Guam than elsewhere in the world (Stone, 1993; Galasko et al., 2002). This was thought to be due to the traditional diet of the people of Guam which included flour made from the seed of a cycad palm (Kurland, 1988; Khabazian et al., 2002; Wilson et al., 2002). Seeds from this cycad are toxic to humans and animals and so are chopped up and washed many times to make them safe for consumption before being made into flour (Kurland, 1988). It has been suggested that toxins within the cycad seed may be responsible for the high incidence of neurological disease in this part of the world (Kurland, 1988; Khabazian et al., 2002; Wilson et al., 2002).

Mice fed flour made from cycad seeds showed significant impairments in motor, olfactory and cognitive abilities along with neuro-degeneration in areas of the brain that were similar to those seen in human ALS-PDC patients (Schulz et al., 2003; Wilson et al., 2002; Wilson et al., 2003; Wilson et al., 2004). Motor functions such as leg extension reflex, gait, and muscle strength and balance were impaired (Shaw & Wilson, 2003), and deficiencies in motor function continued to

worsen even after the feeding of cycad stopped (Wilson et al., 2002). Neurological damage consisted of reductions in the cross sectional areas of parts of the motor and somatosensory cortex, as well as a decrease in the volumes of the hippocampus, substantia nigra, olfactory bulb and spinal cord, and a reduction in dopaminergic terminals in the striatum was also seen (Wilson et al., 2003; Schulz et al., 2003; Shaw & Wilson, 2003; Wilson et al., 2004). Cell death was detected in spinal cord, cortex, hippocampus, substantia nigra and olfactory bulb (Shaw & Wilson, 2003). Spatial learning and reference memory ability were also impaired, as was olfaction (Shaw & Wilson, 2003). Motor impairments were accompanied by loss of motor neurons in spinal cord and decreased thickness in motor cortex (Wilson et al., 2002). Cognitive dysfunctions were accompanied by neuronal cell death in regions of cortex and hippocampus (Wilson et al., 2002). The olfactory bulb was also affected (Wilson et al., 2002).

β-sitosterol-β-D-glucoside (BSSG) was identified as the most likely molecule within the cycad flour to be causing the impairments (Wilson et al., 2002). Both BSSG isolated from cycad flour and synthesised BSSG are toxic to cells from the rat cerebral cortex (Khabazian et al., 2002).

The overall aim of this study is to determine whether the honey bee, *Apis mellifera*, could be used as a model to study the neurological effects of BSSG. The study examines the effects of BSSG on motor behaviour, sucrose sensitivity and olfactory learning in the bee.

1.2 Honey Bee Overview

Honey bees are highly social animals. They live in hives where individual bees work for the colony. The three castes consist of male drones and female queens and workers (Figure 1).

Drone Queen Worker

Figure 1. The castes of the honey bee, *Apis mellifera* (from Gould and Gould, 1988). This study examines worker bees.

Only queens and drones participate in reproduction and they are not involved in work such as foraging for food or colony maintenance (Gould & Gould, 1988; Pankiw & Page, 1999). This study focuses on worker bees. Worker bees perform all of the daily work required to keep the colony thriving, such as building honeycomb, rearing young, cleaning the colony, feeding the queen and drones, guarding the hive, foraging for food, and removing dead bees (Winston, 1987; Gould & Gould, 1988).

1.2.1 Honey Bee Development after Emergence

Worker bee development is not complete at adult emergence (Winston, 1987). During the first twelve to twenty four hours after emergence the cuticle hardens and the body hairs stiffen. Newly-emerged worker bees are soft-bodied, have a fuzzy appearance and are unable to sting until body development is complete. The first five days are crucial for completion of development (Winston, 1987; Fahrbach et al., 2009; Maleszka et al., 2009). Gland development and fat body growth is completed, and both pollen ingestion and nitrogen levels in the body increase during the first five days after emergence (reviewed in Winston, 1987; Page & Peng, 2001). Primary olfactory centres of the brain (the antennal lobes) continue to mature after adult emergence (Wang et al., 2005), and areas of the brain involved in learning and memory (the mushroom bodies) expand during the first few days and continue to increase in volume over the first three weeks of adult life. Evidence suggests that the amount of growth is dependent on the social environment of the bee (Farris et al., 1999; Fahrbach et al., 2009; Maleszka et al., 2009).

1.2.2 Honey Bee Behaviour Changes with Age

Adult worker bees live for four to seven weeks and go through different roles during this time. The first two to three weeks of the life of an adult honey bee is usually spent working within the hive on tasks such as brood care and cell cleaning; older bees four to seven weeks old usually work outside the hive foraging for the colony (Winston, 1987; Gauld & Bolton, 1996; Gould & Gould, 1988; Pankiw & Page, 1999; Schulz & Robinson, 1999; Schulz et al., 2002).

Worker honey bees work inside and outside the hive doing all of the tasks required to keep the colony functioning. However, bees of different ages perform different tasks, with young bees working inside the hive while older ones do the outside jobs (reviewed in Brian, 1983; Winston, 1987; Gould & Gould, 1988; Robinson, 1992; Mercer, 2000; Page & Peng, 2001; Schulz et al., 2002; Elekonich & Roberts, 2005) (Figure 2). Workers progress through the in-hive tasks of cell cleaning, brood and queen rearing, comb building and removing debris from the hive. As they get older bees will perform tasks such as ventilating the hive and guarding the entrance before they become foragers. Although bees generally progress through tasks in this order there is a lot of overlap between tasks and the progression is not a rigid sequence (Winston, 1987).

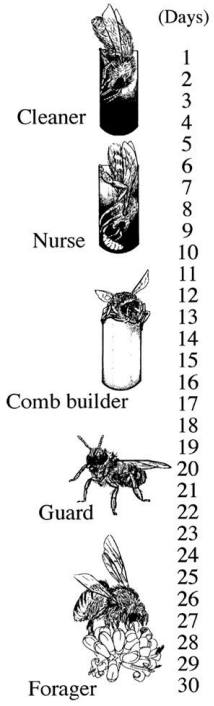

Figure 2. Worker honey bees perform different roles within the colony at different ages (from Mercer, 2000).

Development of the honey bee can be affected by many things throughout adult life. Worker honey bees can change their behavioural state depending on the needs of the colony: they can speed up their development by becoming precocious foragers, delay it by being over-age nurses, or reverse it by returning to nursing after already spending time as a forager (Robinson et al., 1989; Robinson, 1992).

1.2.3 Responsiveness to Sucrose

During a foraging trip a bee learns to associate a particular odour with a food (nectar or pollen) reward. A foraging honey bee may encounter hundreds of flowers in a foraging trip, and as these will vary greatly in the quality of the pollen or nectar produced it is essential for honey bees to be able to discriminate between profitable and un-profitable flowers. Honey bees need to evaporate a lot of water off nectar to store it and this makes it unprofitable to collect nectar with less than 20% sugar except when it will be used immediately, for example to maintain flight (Brian, 1983). Necessarily, honey bees are proficient at detecting low levels of sugar, and taste hairs located on the antennae respond to sucrose concentrations as low as 0.1% (Haupt, 2004). The concentration at which a bee can discriminate between water and sucrose is called the sucrose-response threshold (Page et al., 1998). Foraging bees can detect differences in the sugar concentration of different nectar sources, and this affects their foraging behaviour. Sources with higher concentrations of sucrose lead to more foraging trips, larger loads, and more recruitment of other bees to the resource site (Page et al., 1998). Not all foragers collect the same resource. Some bees will become pollen foragers and some will collect nectar.

Page et al. (1998) studied the sucrose response thresholds of honey bees and found that they differed with respect to whether they were pollen or nectar foragers, whether or not they were hungry, and the genotype of the bees. They found that pollen foragers had lower sucrose-response thresholds than non-pollen foragers (nectar and water foragers) and this was mediated by feeding, which decreased the responsiveness of all bees. The two genotypes were high- and low-pollen-hoarding strains (see Page et al., 1998 for description and citation of origin of bees). High-pollen-strain foragers had lower sucrose-response thresholds and responded more to water than low-pollen-strain foragers. When there were no highly profitable resources high-strain bees would collect nectar with lower concentrations of sugar or water, while low-strain bees would return without collecting anything (Page et al., 1998).

Pankiw and Page (1999) found that differences in response thresholds between high- and low-strain bees were evident as early as zero to two days of age. There were differences in sucrose response thresholds in queens and drones in addition to workers. They concluded that reaction times to sucrose are dependent on genotype and that this affects the foraging behaviour of a bee throughout its life. Scheiner et al. (2001a) also found that genotype affected sucrose perception, but that it had no effect on acquisition or extinction of olfactory conditioning. They showed that a greater number of high-strain bees were able to learn a conditioned stimulus in olfactory and tactile conditioning because they are more responsive to sucrose than low-strain bees. Changes in the pheromones and hormones in the environment can also affect sucrose-response thresholds. Bees raised in the presence of queen mandibular pheromone (QMP) had a higher response threshold than bees raised without QMP, and brood pheromone (BP) raised the sucrose-response threshold of honey bee foragers (Pankiw & Page, 2003). QMP and BP are primer pheromones

and these effects suggest that sucrose sensitivity is a physiological response with a neural basis that can be altered (Pankiw & Page, 2003).

1.2.4 Olfactory Learning and Memory

Efficient foraging involves learning, memory and decision-making. During a foraging trip a honey bee generally focuses on only one species of flower for pollen or nectar gathering (Seeley, 1985). The bees must learn and remember the characteristics of the particular flower they are foraging on such as scent, colour and shape of blossom (Seeley, 1985), as well as the locations of profitable flower patches and the hive (Giurfa, 2003). Profitable flower patches can be unpredictable feeding resources and honey bees can travel over several kilometres on foraging trips (Menzel & Müller, 1996).

Honey bees are good animals for studying learning and memory, as they can be trained to learn many different tasks, and the neural pathways are well known (Menzel, 1983; Menzel & Müller, 1996; Menzel, 1999; Menzel & Giurfa, 2001; Sandoz et al., 2003). The honey bee brain has a volume of $1mm^3$ and contains around 960 000 neurons, yet can perform a wide variety of complex behaviours involving learning and memory, both in the natural environment and in the laboratory (Menzel, 1987; Menzel & Giurfa, 2001; Giurfa, 2003). The simple nervous system of the honey bee compared with vertebrate species makes the task of finding and recording neurons involved in learning and memory much easier in this animal (Menzel, 1983).

Olfaction is often used in studies of learning and memory in honey bees, as bees are good at odour discrimination and the olfactory pathways in honey bees are well known (Winston, 1987;

Menzel & Müller, 1996; Menzel, 1999; Giurfa, 2003). The olfactory structures of the honey bee are the two antennae located on the top of the head; these are multifunctional organs containing olfactory, gustatory, humidity, temperature and mechanosensory receptors (Winston, 1987; Resch et al., 1998; Haupt, 2004; de Brito Sanchez et al., 2007). Each antenna is divided into three sections: the scape, pedicel and flagellum (Figure 3). The overall sensitivity of the olfactory system is similar to humans but worker bees are ten to one hundred times more sensitive to biologically important odours such as wax and flower odours, and can also detect the direction of an odour by comparing the odour molecule intensity perceived by each antenna (reviewed in Winston, 1987).

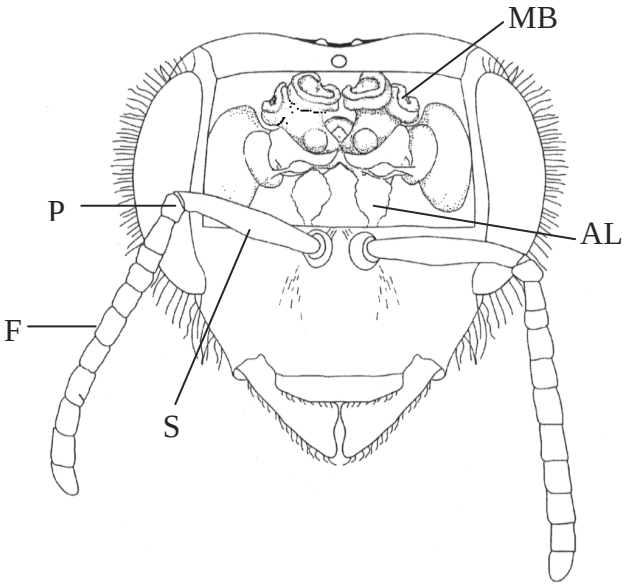

Figure 3. Diagram of the head and brain of an adult worker honey bee. A window has been cut in the anterior surface of the head capsule allowing the brain to be seen (modified from Mercer, 2000). MB, Mushroom Body; AL, Antennal Lobe; P, Pedicel; F, Flagellum; S, Scape.

The main olfactory regions of the brain are the antennal lobes and mushroom bodies (Figure 3). Olfactory information is received by the antennae and sent to the antennal lobes. The mushroom bodies are higher-order centres in the brain that integrate olfactory, visual and mechanosensory information (reviewed in Menzel & Müller, 1996; Menzel, 1999; Giurfa, 2003). A honey bee will quickly learn an association between sucrose used as a reward and an initially neutral stimulus, which after being paired with sucrose elicits a conditioned response from the bee. There are, however, several factors that can influence how well bees will learn an association. Ray and Ferneyhough (1999) showed that learning ability in honey bees may be dependent on the behavioural role performed within the colony. They demonstrated that although foragers were highly successful at acquisition and retention during olfactory conditioning, nurse bees did not perform as well as foragers in this task. However, precocious foragers showed high levels of learning even though they were the same biological age as the nurse bees tested, indicating that behavioural role is more important for learning than age in honey bees.

During olfactory conditioning information relative to the odour and the sucrose reward are integrated in the antennal lobes and mushroom bodies (Menzel, 2001; Déglise et al., 2003). Chilling the antennal lobes and/or the mushroom bodies immediately after single-trial conditioning induces retrograde amnesia, and replacing the sucrose reward with octopamine injection into either the antennal lobes or mushroom body calyces leads to olfactory conditioning (reviewed in Menzel, 1999; Menzel, 2001).

1.2.5 Biogenic Amines are Important for Learning, Memory and Motor Control

In insects, as in mammals, biogenic amines play a role not only in learning and memory, but also motor control (Mercer & Menzel, 1982; Menzel, 1983; Macmillan & Mercer, 1987; Farooqui et al., 2003; Schwaerzel et al., 2003; Schroll et al., 2006). In bees, amine levels change with age; newly emerged worker bees have significantly lower octopamine, dopamine and serotonin levels than older bees, and amine levels have been correlated with behavioural state (Harris and Woodring, 1992; Taylor et al., 1992; Schulz & Robinson, 1999; Wagener-Hulme et al., 1999; Schulz et al., 2002). Worker age and behavioural state can be uncoupled. For example, worker bees can be induced to change their behavioural state by changing the proportions of workers in the colony (Robinson et al., 1989; Wagener-Hulme et al., 1999). Bees will become precocious foragers if there are no older bees in the colony, and foragers will revert to nursing duties if there are no young adults, as nurses are needed to care for the young. Regardless of age, levels of juvenile hormone have been shown to be higher in foragers than nurses (Robinson et al., 1989). Wagener-Hulme et al. (1999) found higher octopamine levels in foragers than nurses during comparisons between precocious foragers and normal-age nurses, normal-age foragers and overage nurses, but not for foragers and reverted nurses. They found age-related differences for dopamine, but not for octopamine or serotonin. Wagener-Hulme et al. (1999) concluded that age and previous experience influenced dopamine and serotonin levels, while high octopamine levels were associated with foraging. In a similar experiment Schulz and Robinson (1999) compared the levels of dopamine, serotonin and octopamine in the antennal lobes and mushroom bodies of the brains of honey bees of different ages and behavioural states. They found that in the mushroom bodies all three amines varied with respect to age, but not behaviour. Older bees had higher levels of dopamine, octopamine and serotonin regardless of behavioural state.

Conversely, in the antennal lobes all three amines varied with behaviour but not age, with foragers having higher levels than nurses, and the difference was greatest with octopamine. Schulz and Robinson (1999) concluded that levels of octopamine in the antennal lobes were a significant aspect of age-related division of labour.

Dopamine in insects plays a critical role in aversive learning (Schwaerzel et al., 2003; Vergoz et al., 2007), and can inhibit the retrieval of appetitive memories (Mercer and Menzel, 1982; Menzel, 1983). Octopamine, on the other hand, has been strongly implicated in the formation of appetitive memories (reviewed in Menzel, 1983; Hammer, 1997). Farooqui et al. (2003) found that disrupting receptors in the octopamine pathway of the antennal lobes impaired acquisition and lowered responses to a conditioned odour, but had no effect on odour discrimination. They concluded that octopamine in the antennal lobes forms a link between an olfactory conditioned stimulus (CS) (odour) and the unconditioned stimulus (US) (sucrose). Injecting octopamine into the calyces of the mushroom bodies instead of presenting a sucrose reward to a honey bee during conditioning with multiple trials leads to learning (reviewed in Menzel, 1983; Hammer, 1997). Schwaerzel et al. (2003) demonstrated that dopamine and octopamine, respectively, are required for the formation of aversive and appetitive olfactory memories in the fly *Drosophila melanogaster*. They used the same odour to condition an appetitive (sugar) and aversive (electric shock) US, and found that the mushroom bodies were important for both types of conditioning. In addition to this, octopamine was required for appetitive learning and dopamine was required for aversive learning. Blocking either dopamine or octopamine specifically impaired one type of learning, but not the other (Schwaerzel et al., 2003).

1.3 Aims and Hypotheses of the Present Study

In mammals, dopamine pathways are affected by BSSG (see Section 1.1 above). If this is true also in honey bees, then treatment with BSSG should have a significant effect on locomotor activity, as well as on learning and memory in the bee (see Section 1.2, above). This study investigates the effects of BSSG on worker bee motor behaviour, sucrose sensitivity and olfactory learning and memory. The aims of this study are summarised as follows:

1. To examine the effects of BSSG on motor behaviour in young adult worker bees.
2. To examine the effects of BSSG on sucrose sensitivity.
3. To examine the effects of BSSG on associative learning and memory.
4. To compare the effects of BSSG with effects of paraquat, an herbicide known to affect motor control in many animals.

Based on the effects of BSSG observed in mammals and effects of paraquat on insects described above, it is hypothesised that:

1. Bees fed BSSG will show impairments in motor behaviour relative to controls.
2. Bees fed BSSG will show impairments in sucrose sensitivity relative to controls.
3. Bees fed BSSG will show impairments in associative learning and memory relative to controls.
4. Bees fed paraquat will show impairments in motor behaviour, sucrose sensitivity and associative learning and memory compared with controls.

5. Impairments in motor behaviour, sucrose sensitivity and learning and memory in bees fed BSSG will be similar to impairments in bees fed paraquat.

2. METHODS

2.1 Animal Acquisition

The animals used for this research were honey bees, *Apis mellifera*. They were gathered from hives in the Department of Zoology during the period encompassing the spring of 2005 to the autumn of 2006. When weather conditions were right the hives were opened and frames were checked for brood. If a frame had enough newly-emerging brood it was removed from the hive and the brood were allowed to develop in an incubator.

2.2 Incubation

Brood frames were placed into an incubator set to 35°C, as this is the temperature found inside the hive (Brian, 1983; Bujok et al., 2002, Tautz et al., 2003). Frames were cleared of emerging brood twice a day in the morning and evening, and the honey bees that had emerged during the night were used. Bees were placed into small plastic cages with holes in the top and bottom, and with a separate chamber for food (Figure 4). The cages were placed over a trough of water in an incubator set to 35°C and bees were able to drink by sticking their proboscis through the holes in the bottom of the cage. There was a gap between the main part of the cage and the food chamber to allow the bees to feed from pollen patties placed in the food chamber.

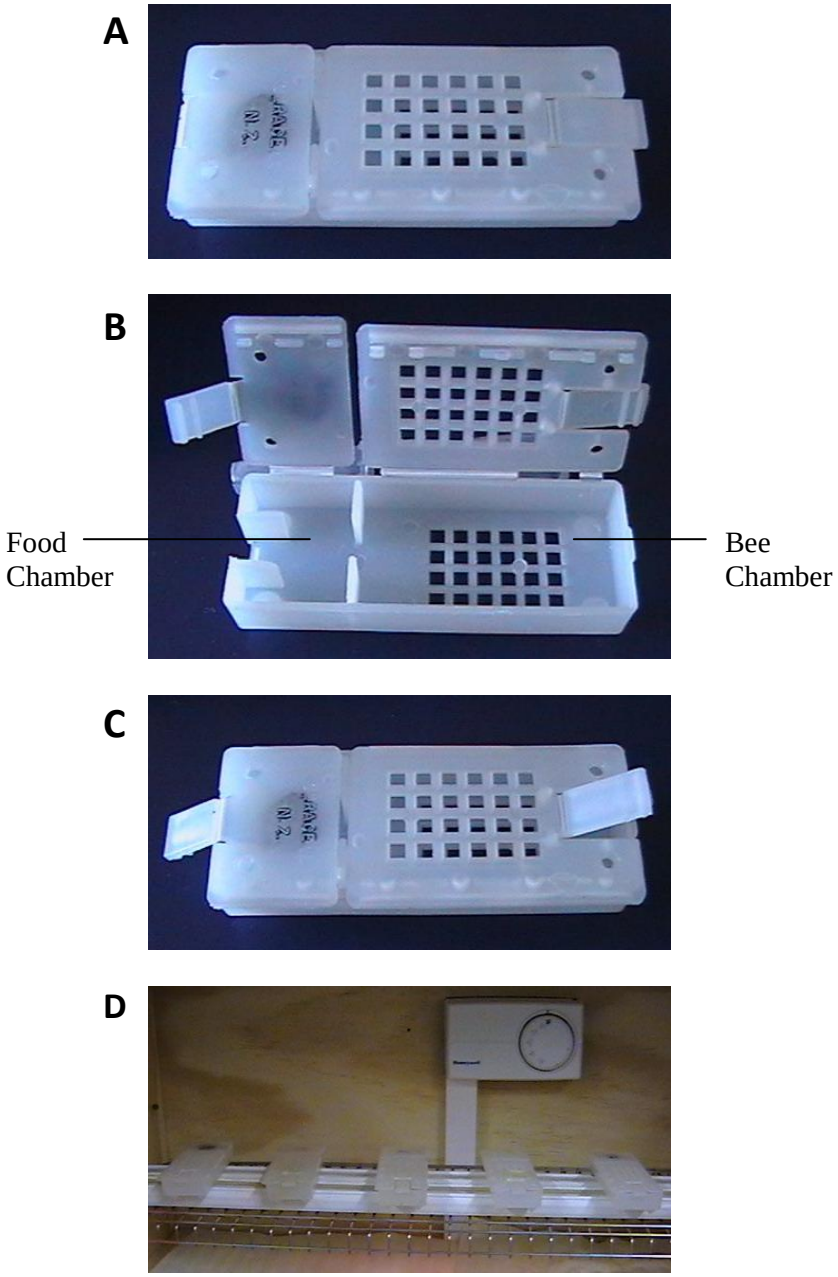

Figure 4. Bee cages used within incubators. **A.** All doors closed to prevent bees from escaping. **B.** Open bee cage showing separate chambers for bees and food. **C.** Main compartments closed but small doors at either end open. These doors allowed individual bees to be removed without disturbing the rest of the bees within the cage. **D.** Bee cages inside incubator. Honey bees inside the cages were able to extend their proboscis through holes in the bottom to drink fresh water from the trough underneath.

2.3 Paraquat Used as a 'Control'

In this study paraquat was used to determine whether compounds toxic to bees added to food are ingested, or whether bees avoid toxin-laced foods. Paraquat is an herbicide that is toxic to many species, and the risk of developing Parkinson's disease is greater for people who have been exposed to herbicides such as paraquat (Brooks et al., 1999; Shimizu et al., 2003; Uversky, 2004). Paraquat exposure also results in an increase of α-synuclein in the brain; something that is seen in age-related neurological diseases such as Parkinson's disease (Manning-Bog et al., 2002). Brooks et al. (1999) demonstrated that administering paraquat to mice led to a reduction of dopaminergic neurons in the substantia nigra and of dopaminergic nerve terminals in the striatum, as well as decreasing the locomotor activity of the mice.

2.4 Toxin Administration and Experimental Groups

BSSG was administered to bees in food, in the form of pollen patties. Pollen patties were made up of a mixture of pollen granules, sugar, brewer's yeast, lactalbumin, and water (Appendix A). BSSG was made up into a 2 M stock solution with sesame oil. No adjustment for density was made. This stock solution was then used to make a pollen patty containing 20 mM of BSSG. This was further diluted by adding untreated pollen patty mix to produce pollen patties containing 20 nM, 2 μM or 200 μM BSSG. These concentrations were calculated to be proportional to the doses of BSSG administered to mice in previous studies, adjusted to compensate for the much smaller size of honey bees. Paraquat was made up into a 1 M stock solution with water. This stock solution was then used to make a concentrated pollen patty mix containing 10 mM

paraquat, which was diluted using untreated pollen patty mix to give a final concentration of 100 μM paraquat. This dose of paraquat is the same as the highest dose of BSSG used, and was chosen so that effects of the two toxins could be compared directly. Bees in a control group were fed pollen patties containing neither paraquat nor BSSG. The amount of pollen patty consumed by each bee was not recorded. Attempts were made to weigh the food after treatment in order to calculate the amount of food ingested by each group of bees. However, the amount of food ingested by each group was small, and the sticky quality of pollen patties resulted in food sticking to the sides of the cages. Therefore it was felt that measurements of this kind would be too inaccurate.

Five experimental conditions were tested concurrently: three BSSG groups (20 nM, 2 μM, 200 μM), one paraquat group (100 μM) and a control group fed on untreated pollen patties (no toxin treatment).

2.5 Motor Experiments

Motor ability was tested using three methods: a grid line test, time to right, and a climbing test. Motor experiments were performed when bees were one-day, four-days and six-days old. On any day that motor experiments were performed they were carried out for all five of the treatment groups and were always executed in the morning. This was to avoid any confounding results from possible differences between brood frames, or other daily variables. The motor experiments were conducted at an ambient temperature of approximately 20°C.

The *grid line test* was used as a measure of the bees' activity. A similar test was used by Humphries et al. (2005) to measure activity in newly-emerged bees. Individual bees were placed under a clean petri dish lid marked with grid lines on the top (Figure 5). The lid was 1cm high to prevent bees from flying, and had a diameter of 14.5cm. Grid lines were 2cm apart, resulting in squares of 2cm by 2cm. Bees were placed under the lid and timed for two minutes. During this time each grid line crossed was counted using a hand-held counter. A line cross was counted if the middle of the abdomen passed the line in the direction the bee was moving. Grid lines were not counted if the abdomen passed a grid line in the process of the bee turning around to change direction. Data are expressed as the mean number of grid lines crossed within a two-minute period.

Figure 5. The grid line test apparatus consisted of a petri dish lid with vertical and horizontal lines marked on top.

The *time to right* test was a measure of the bees' coordination and balance. Bees were flipped onto their backs and the time it took them to right themselves was recorded (Figure 6). If a bee had not righted itself after thirty seconds had passed the timer was stopped and a time of thirty seconds was recorded. The test was run three times per bee and the mean time was calculated and used as the overall time for that bee.

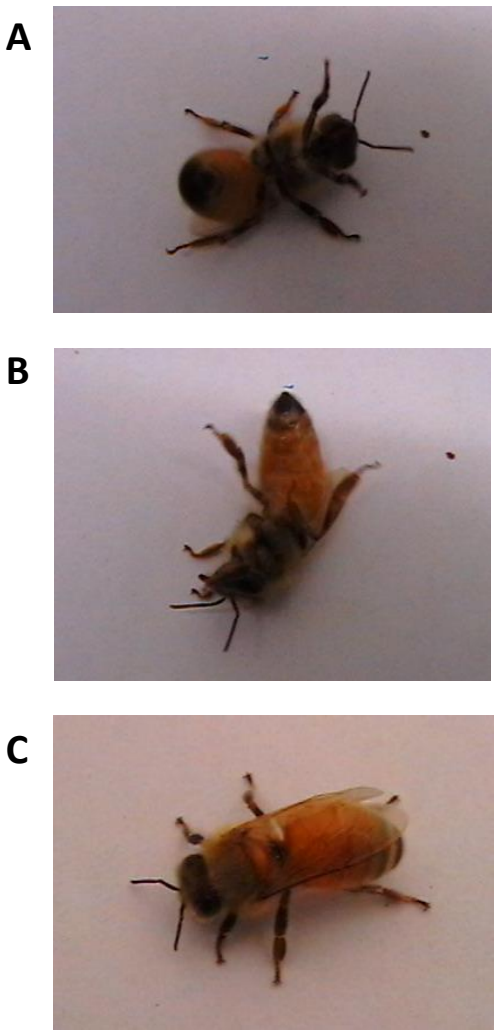

Figure 6. Honey bee during the time to right test. **A.** The bee after it has been flipped onto its back. **B.** The bee in the process of righting. **C.** The honey bee upright after righting itself.

The third test used to examine motor behaviour was a *climbing test*. Individual bees were placed into the bottom of a large measuring cylinder and timed for one minute (Figure 7). The maximum height climbed by each bee was recorded, as well as the number of times it fell off the wall of the cylinder, and the number of flying attempts observed. Also noted were any problems the bee had getting onto the wall of the cylinder. This was recorded as the number of trips, and was defined as the number of times a bee tried to climb onto the wall and slipped off to one side with at least the back pair of legs still on the floor of the cylinder.

Figure 7. Apparatus for the climbing test. **A.** Empty cylinder large enough to allow bees to fly off the wall. **B.** Climbing cylinder with bee on wall.

2.6 Preparation of Bees for Sensory and Conditioning Experiments

Bees were placed into jars and put in a container of ice to lower the body temperature and reduce movement. Once the bees had stopped moving they were removed from the ice and mounted in small plastic Eppendorf tubes (Figure 8). This was done by placing a bee into the tube so that the head was sticking out of the top. A thin strip of cardboard was then placed between the head and thorax and secured with tape so the head was immobilized. This ensured that the bee could only move its antennae and proboscis. Each tube was then placed upright onto a board and secured.

Figure 8. Honey bee mounted in an Eppendorf tube. The head is immobilised but the bee is able to move its antennae and proboscis.

Once the bees were mounted in the Eppendorf tubes they were left for thirty minutes to recover from the refrigeration and mounting procedure. On any day that sensory or learning experiments were done they were done for all five of the treatment groups and were always executed in the afternoon for sensory experiments, and during the middle of the day for learning experiments. This was to avoid any possible confounding results from changing daily variables. Sensory and

learning experiments were done at an ambient temperature of approximately 20°C. There was some difficulty in obtaining sufficient numbers of bees for sensory and conditioning experiments, as the longer the bees were kept in cages, the less likely they were to survive.

2.7 Sucrose Sensitivity Measurements

When the bees were seven days old their sucrose sensitivity was tested by presenting bees with varying concentrations of sucrose solution. Five sucrose concentrations were used: 0.1%, 3%, 10%, 30% and 55%. Sucrose solutions were presented in ascending order of concentration with a presentation of water at the beginning, the end, and between each solution, and with an inter-stimulus interval of five minutes between each presentation of either sucrose or water. Sucrose and water solutions were presented by holding a drop of solution close to the bees' antennae. If a bee detected the sucrose it extended its proboscis in anticipation of food. Neither sucrose nor water was ever touched on the antennae or fed to the bees during the experiment. This was to avoid the possibility of sucrose remaining on the antennae and leading to sensitisation of the bees to sucrose. Whether bees responded to sucrose and/or water presentations was recorded for each treatment.

2.8 Learning Experiments

Bees that were eight days old had their learning ability tested using one-trial olfactory conditioning. This technique uses Pavlovian conditioning, a paradigm that has frequently been

used in tests of learning and memory in bees (Menzel, 1983; Ray & Ferneyhough, 1997; Ben-Shahar & Robinson, 2001; Behrends, et al., 2007; Arenas & Farina, 2008). An unconditioned stimulus (US) produces an unconditioned (reflex) response (UR). The US is paired with a conditioned stimulus (CS), which is initially neutral. After several pairings of CS and US, the CS begins to elicit a conditioned response (CR), which is very similar to the UR. In honey bees, stimulation of the antennae by touching it with a drop of sucrose results in the bee extending its proboscis, called the proboscis extension reflex (PER). Olfactory conditioning in honey bees consists of pairing an odour (CS) with a sucrose reward (US) to elicit the PER. If the bee learns to associate the odour with a food reward it will respond with proboscis extension (CR) to the odour alone, in the absence of a food reward.

In this study, a eugenol scent (CS) was paired with a sucrose reward (US). The eugenol scent was prepared by placing a few drops of eugenol oil concentrate onto a small piece of paper and placing the paper into a plastic syringe. The scent was then puffed over the bees' antennae for a controlled amount of time (four seconds). Checking for spontaneous responses to eugenol, conditioning, and testing were all done under an extractor pipe to prevent the scent from lingering in the air.

Prior to conditioning a drop of sucrose solution was lightly touched to the bees' antennae to ensure they responded to sucrose. Any bees not responding to sucrose were eliminated from the experiment (5.7% and 0.5% for 10% and 55% sucrose concentrations, respectively). The bees were then left for thirty minutes, after which the eugenol scent was puffed over the antennae of each bee to determine whether or not there was any response to the scent alone. Any bees that responded by extending their proboscis in response to the scent prior to conditioning were also

eliminated from the experiment (0.4%). The bees were left for another thirty minutes to ensure the smell of eugenol was not still present. During conditioning the eugenol scent was puffed over the antennae for four seconds. After two seconds one antenna was touched with a drop of sucrose solution at either 10% or 55% concentration to motivate the bee to extend its proboscis. The bee was then allowed to feed on the sucrose solution for one to two seconds.

After conditioning the bees were left for one hour, before being tested to determine whether associative learning had occurred. Testing consisted of the eugenol scent being puffed over the antennae of each bee for two to three seconds. The odour was presented alone, without the sucrose solution. If a bee extended its proboscis to the eugenol scent without obtaining a reward then learning had occurred, as the bee was associating the odour with the food reward even though the reward was absent. The number of bees that extended their proboscis for each treatment group during testing was recorded. Two separate learning experiments were performed; one used a 10% sucrose solution as the reward and the other used a 55% solution. The 10% solution was used as this concentration has been successfully used in other learning studies (Scheiner et al., 2001a; Scheiner et al., 2001b). The learning experiment was then repeated using a 55% solution to guard against the possibility that sucrose sensitivity might be affected by paraquat and/or BSSG. If this was the case more bees would be likely to respond to the higher concentration of sucrose and thus it would be easier to separate any possible learning deficits from sucrose sensitivity impairments.

2.9 Statistical Analysis

A minimum of thirty bees was used for each age and treatment group for all experiments, with the exception of the learning experiment with 10% sucrose solution as the reward. The numbers for this experiment were not quite thirty due to time constraints and problems obtaining sufficient bees and keeping them alive. In cases where more than one test was performed on a data set the critical p-value was adjusted using a Bonferroni correction to ensure the overall critical p-value for the data stayed at least at the 95%, or 0.05, level of significance.

For most of the tests used here two p-values were available: a normal p-value and a p-value adjusted for ties. It was not necessary to use both p-values, so to ensure consistency the same p-value was used for all statistical tests. In all cases both p-values were looked at but the p-value adjusted for ties was used, as the data sets did contain tied data. It should be noted that the p-value adjusted for ties is higher if there are several ties in the data, and so it is a more conservative estimate of the p-value (Dytham, 2003). For most of the tests the adjusted and non-adjusted for ties p-value were either both significant or both not significant. There were, however, a few cases where one value was significant and one was non-significant. In these cases the adjusted for ties p-value has been used as it is the more conservative estimate and also to ensure consistency with the rest of the tests.

2.9.1 Motor Experiments

2.9.1. i Control

The Kolmogorov-Smirnov test was performed to check if the data followed a normal distribution. For all motor tests the data were significantly different from normal, so the data were logged to try and bring the distribution closer to that of a normal distribution. The Kolmogorov-Smirnov test was then performed on the logged data to check for normality. For all but one of the motor tests the data were still significantly different from normal; the test for falls was the exception. There was no need to check for heterogeneity as the data were not distributed normally. Non-parametric tests were used to analyse the untransformed data for all of the motor tests, including the falls data. The falls data were analysed using non-parametric tests to keep consistency with the other tests as the non-transformed data were not normally distributed.

The Kruskal-Wallis test was performed to test for significance. If a significant difference was found a post-hoc test was necessary to determine which groups were significantly different from each other. Pairwise Mann-Whitney U tests were used for this and the critical p-value was adjusted to ensure the overall critical p-value was at the 0.05 level of significance.

2.9.1. ii Paraquat and BSSG

The Kolmogorov-Smirnov test was performed to check if the data followed a normal distribution. For all motor tests the data were significantly different from normal, and so were logged to try and bring them closer to a normal distribution. The Kolmogorov-Smirnov test was then

performed on the logged data to check for normality. For all of the motor tests the data were still significantly different from normal. For this reason non-parametric tests were used to analyse the untransformed data.

For each treatment condition of paraquat and BSSG a Kruskal-Wallis test was used to examine the data across the three age groups. If the Kruskal-Wallis test was significant, pairwise Mann-Whitney U tests were used as a post-hoc analysis and the critical p-value adjusted to compensate for the number of tests.

2.9.1. iii Control vs Treatment

Control vs paraquat data were analysed using a Mann-Whitney U test on each age group. For control vs BSSG data, overall statistical significance was determined by a Kruskal-Wallis test at each age. If a significant difference was found pairwise Mann-Whitney U tests were used as post-hoc comparisons and the critical p-value was adjusted to ensure the overall critical p-value remained at the 0.05 level of significance.

2.9.1. iv Behavioural Development

As control and all treatment conditions showed the same trends and similar significant effects, the data were pooled and examined as one data set. Kruskal-Wallis tests were used to examine the overall age data for each behavioural assay and pairwise Mann-Whitney U tests were used as post-hoc comparisons. The exception to this was flight attempt data, as all values in the one-day old group equaled zero; Mann-Whitney U tests are unable to be performed on a data set where all

the values are equal. Dytham (2003) states that Mann-Whitney U tests are usually preferred when two samples are being analysed as it is the more powerful test with two samples, but Kruskal-Wallis tests may still be used. Therefore, Kruskal-Wallis tests were used as post-hoc comparisons for the flight attempt data.

2.9.2 Sucrose Sensitivity Measurements

The Kolmogorov-Smirnov test was performed to check if the data followed a normal distribution. All data sets were significantly different from normal, so the data were logged and then a Kolmogorov-Smirnov test was performed on the logged data. All data sets were still significantly different from a normal distribution, therefore a non-parametric test was needed to analyse the data.

Kruskal-Wallis tests were used to look at responses to sucrose and water within each treatment group to examine if there was a significant change in the level of responses to sucrose as the concentration increased, and also to determine whether sensitisation was occurring.

Kruskal-Wallis tests were also used to determine the sucrose response threshold for each treatment group, defined as the concentration of sucrose where responses were significantly greater than initial responses to water. As many of these data sets contained identical data (for example where responses to water all equaled zero) Mann-Whitney U tests were unable to be used for these data sets.

Kruskal-Wallis tests were then performed at 30% and 55% sucrose concentrations as earlier tests indicated these concentrations were equal to or greater than the sucrose response thresholds of most of the treatment groups. Mann-Whitney U tests were performed as post-hoc analysis comparing each treatment against the control group and the critical p-value was adjusted to compensate for the increased number of tests.

2.9.3 Learning Experiments

For both of the learning experiments Chi-square tests were used to compare each treatment group to the control group and the critical p-value was adjusted to keep the overall significance level of the tests at the 0.05 level.

3. RESULTS

3.1 Effects of β-Sitosterol-β-D-Glucoside (BSSG) and Paraquat on Motor Activity

3.1.1 β-Sitosterol-β-D-Glucoside (BSSG): Effects on Motor Activity

BSSG was administered to honey bees in food at concentrations of 20 nM, 2 μM and 200 μM. Activity was not affected by BSSG at any of the concentrations used in this study (Figure 9a). There was also no difference in righting behaviour between the treatment groups at ages of one and four days (Figure 9bi,ii), although surprisingly, at six days of age control bees took significantly longer to right than bees treated with BSSG at a concentration of 20 nM ($p<0.005$) (Figure 9biii).

Climbing ability was not affected by BSSG, as there were no significant differences between control bees and bees treated with BSSG at any of the concentrations tested, in the height climbed (Figure 10a), how often they fell (Figure 10b), or the bees' ability to get onto the wall of the climbing cylinder (Figure 11a). There was no difference between the groups in the number of flying attempts made at six days of age, but the Kruskal-Wallis test returned a significant difference at four days of age ($p<0.05$) (Figure 11b). Mann-Whitney U tests used as a post-hoc comparison indicated a trend towards bees treated with BSSG 200 μM making fewer flying attempts than control bees, but as the critical p-value was adjusted to compensate for the number of tests this difference was not found to be significant.

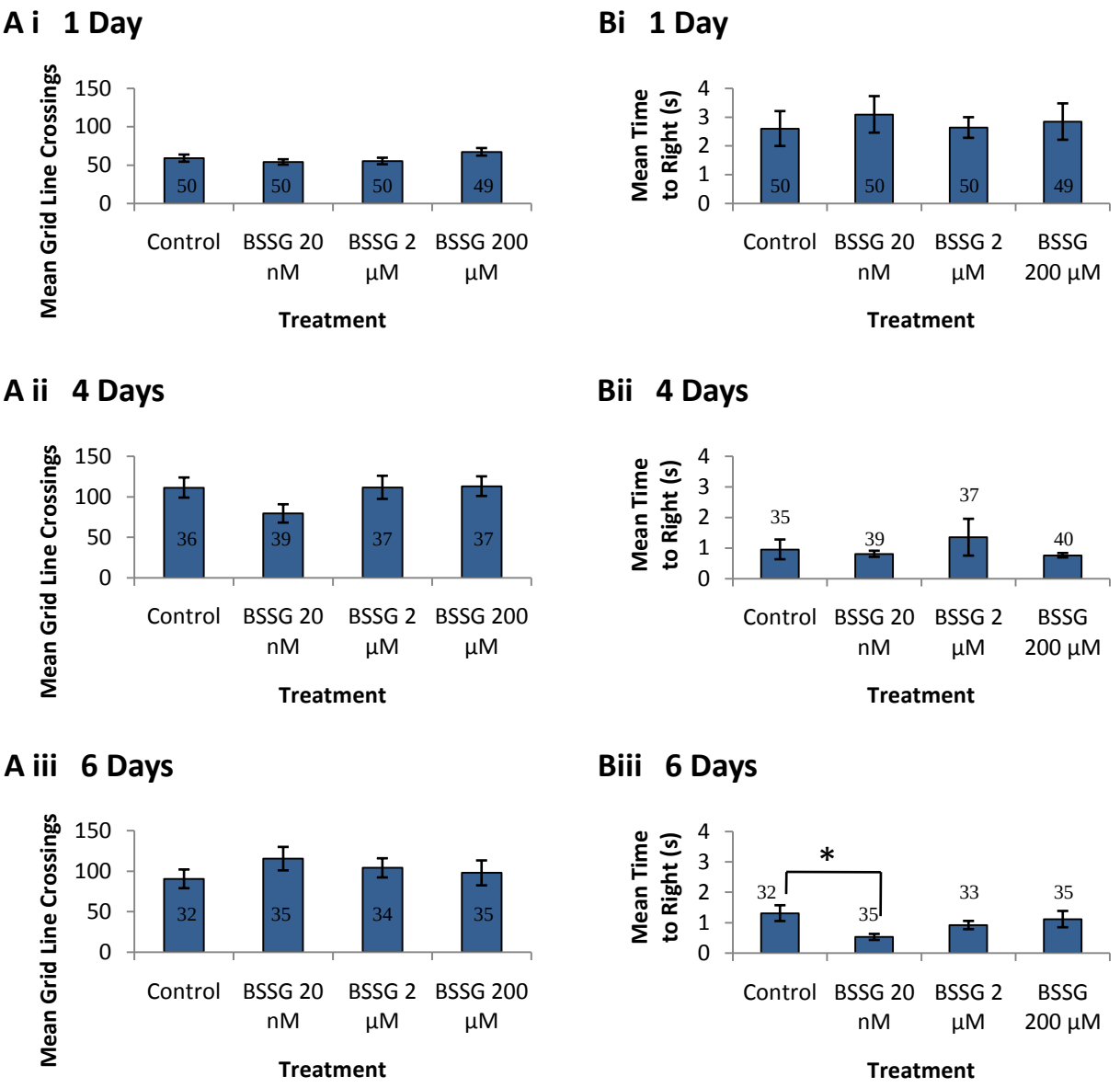

Figure 9. Numbers of bees tested in each group are shown in or above columns. **A.** Activity levels. Mean number of times (±S.E.) one-, four- and six-day old control and BSSG-treated bees crossed grid lines over a two-minute period during the grid line test. Statistical significance was determined by Kruskal-Wallis test. **Ai.** 1 day ($p=0.167$). **Aii.** 4 days ($p=0.185$). **Aiii.** 6 days ($p=0.577$). **B.** Righting behaviour. Mean time taken (in seconds) (±S.E.) for one-, four- and six-day old control and BSSG-treated bees to right themselves after being flipped onto their backs. The asterisk indicates a significant difference between the groups. Statistical significance was determined by Kruskal-Wallis test followed by pairwise Mann-Whitney U tests for post-hoc comparisons. **Bi.** 1 day ($p=0.678$). **Bii.** 4 days ($p=0.808$). **Biii.** 6 days ($p<0.05$: control vs 20 nM $p<0.005$; control vs 2 µM $p=0.3138$; control vs 200 µM $p=0.2920$; 20 nM vs 2 µM $p=0.0174$ [adjusted critical $p=0.0083$]; 20 nM vs 200 µM $p=0.0523$; 2 µM vs 200 µM $p=0.8762$).

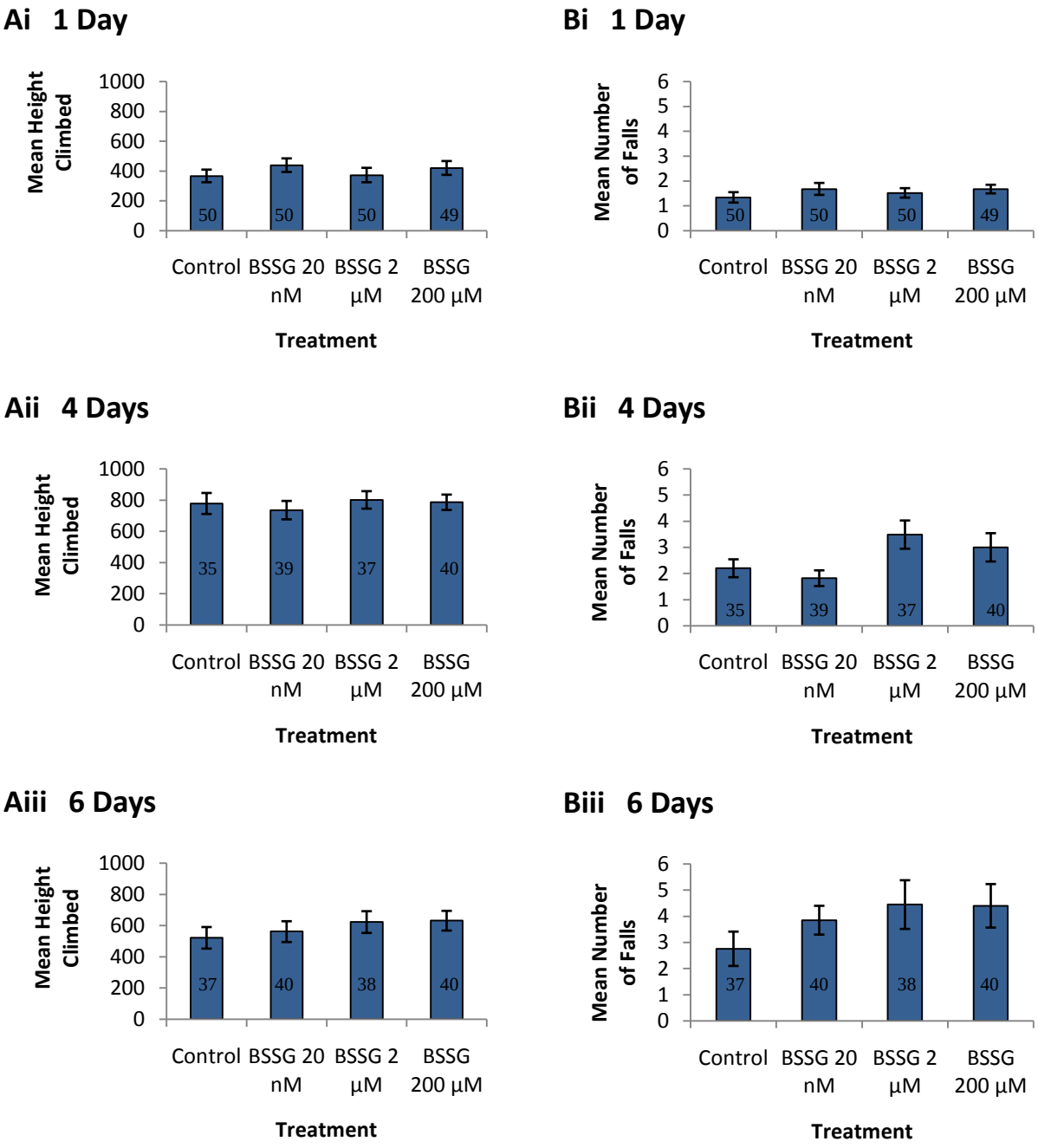

Figure 10. Numbers of bees tested in each group are shown in columns. **A.** Climbing ability. Mean height climbed (±S.E.) by one-, four- and six-day old control and BSSG-treated bees over a one-minute period during the climbing test. Statistical significance was determined by Kruskal-Wallis test. **Ai.** 1 day (p=0.432). **Aii.** 4 days (p=0.711). **Aiii.** 6 days (p=0.631). **B.** Falls. Mean number of times (±S.E.) one-, four- and six-day old control and BSSG-treated bees fell off the wall of the climbing cylinder over a one-minute period. Statistical significance was determined by Kruskal-Wallis test. **Bi.** 1 day (p=0.359). **Bii.** 4 days (p=0.056). **Biii.** 6 days (p=0.238).

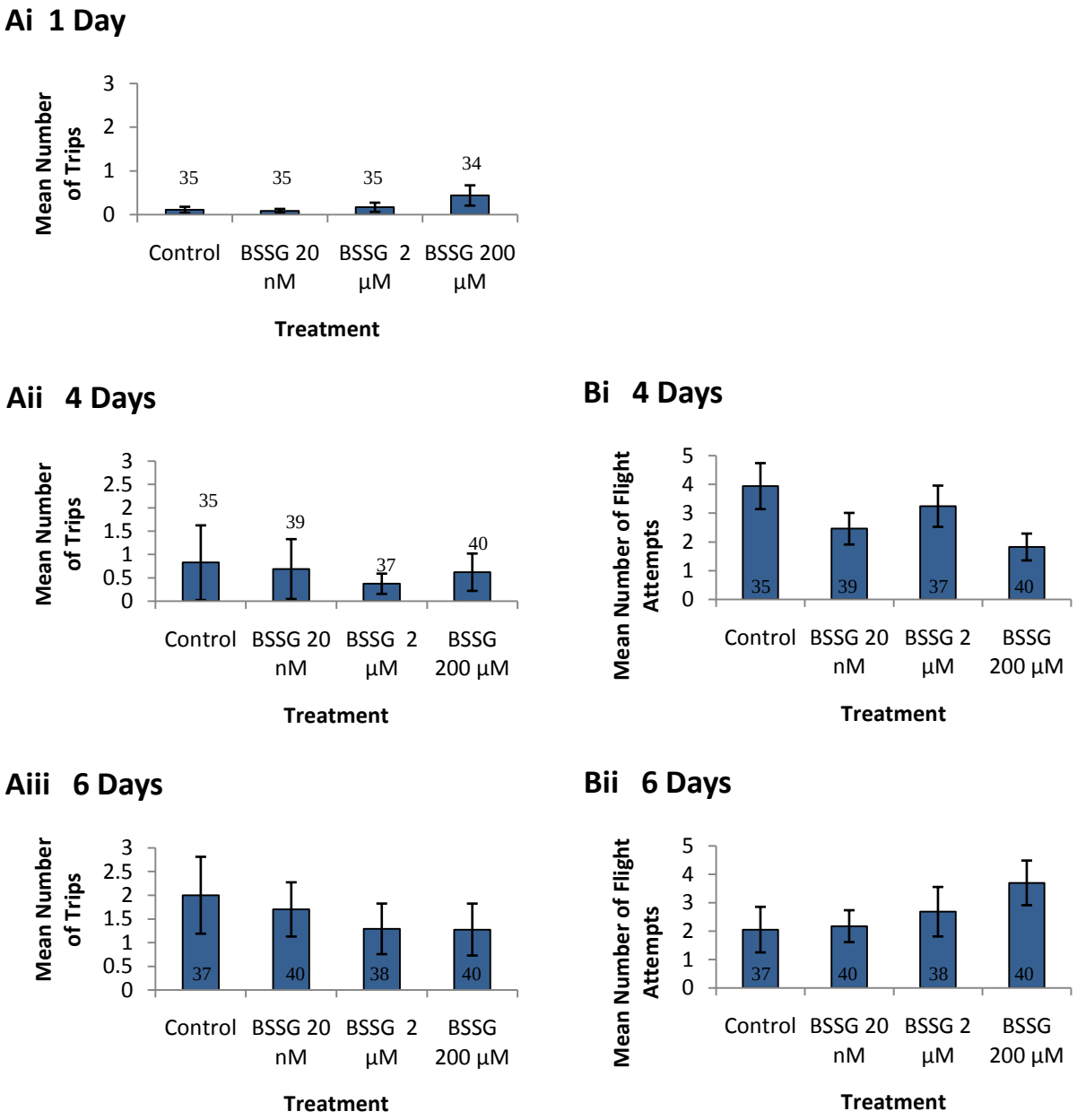

Figure 11. Numbers of bees tested in each group are shown in or above columns. **A.** Trips. Mean number of trips (±S.E.) made by one-, four- and six-day old control and BSSG-treated bees over a one-minute period during the climbing test. A trip was defined as an unsuccessful attempt made by a honey bee to climb onto the wall of the climbing cylinder where it slipped off to one side with at least the back pair of legs still on the floor of the cylinder. Statistical significance was determined by Kruskal-Wallis test followed by Mann-Whitney U tests for post-hoc comparisons. **Ai.** 1 day (p=0.745). **Aii.** 4 days (p=0.867). **Aiii.** 6 days (p=0.920). **B.** Flight attempts. Mean number of flight attempts made by one-, four- and six-day old control and BSSG-

3.1.2 Paraquat Effects on Motor Activity

Paraquat was administered in food at a concentration of 100 μM to determine if it had an effect on the motor behaviour of young (less than one week old) honey bees. The control data are presented again with paraquat as it was the comparison between the toxin treatments and the control that was being examined. Paraquat (100 μM) had no effect on activity levels regardless of age (Figure 12a), but at one day of age bees treated with paraquat took longer to right themselves than control bees ($p<0.05$) (Figure 12bi). There was no difference in righting behaviour at either of the other two ages tested (Figure 12bii,iii).

Paraquat did not affect the climbing ability of young honey bees, as control and paraquat-treated bees did not differ in the height climbed (Figure 13a), how often they fell off the wall (Figure 13b), or their ability to get onto the wall of the climbing cylinder (Figure 14a). However, at four days of age control bees made more flying attempts than paraquat-treated bees ($p<0.05$) and at six days of age paraquat-treated bees made more flying attempts than control bees ($p<0.05$) (Figure 14b).

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treated bees over a one-minute period during the climbing test. Data from one-day old bees has not been shown as all values were equal to zero ($p=1.000$). Statistical significance was determined by Kruskal-Wallis test followed by Mann-Whitney U tests for post-hoc comparisons. **Bi.** 4 days ($p<0.05$: Control vs 20 nM $p=0.1005$; control vs 2 μM $p=0.5379$; control vs 200 μM $p=0.0101$ [adjusted critical $p=0.0083$]; 20 nM vs 2 μM $p=0.2503$; 20 nM vs 200 μM $p=0.4498$; 2 μM vs 200 μM $p=0.0371$ [adjusted critical $p=0.0083$]). **Bii.** 6 days ($p=0.278$).

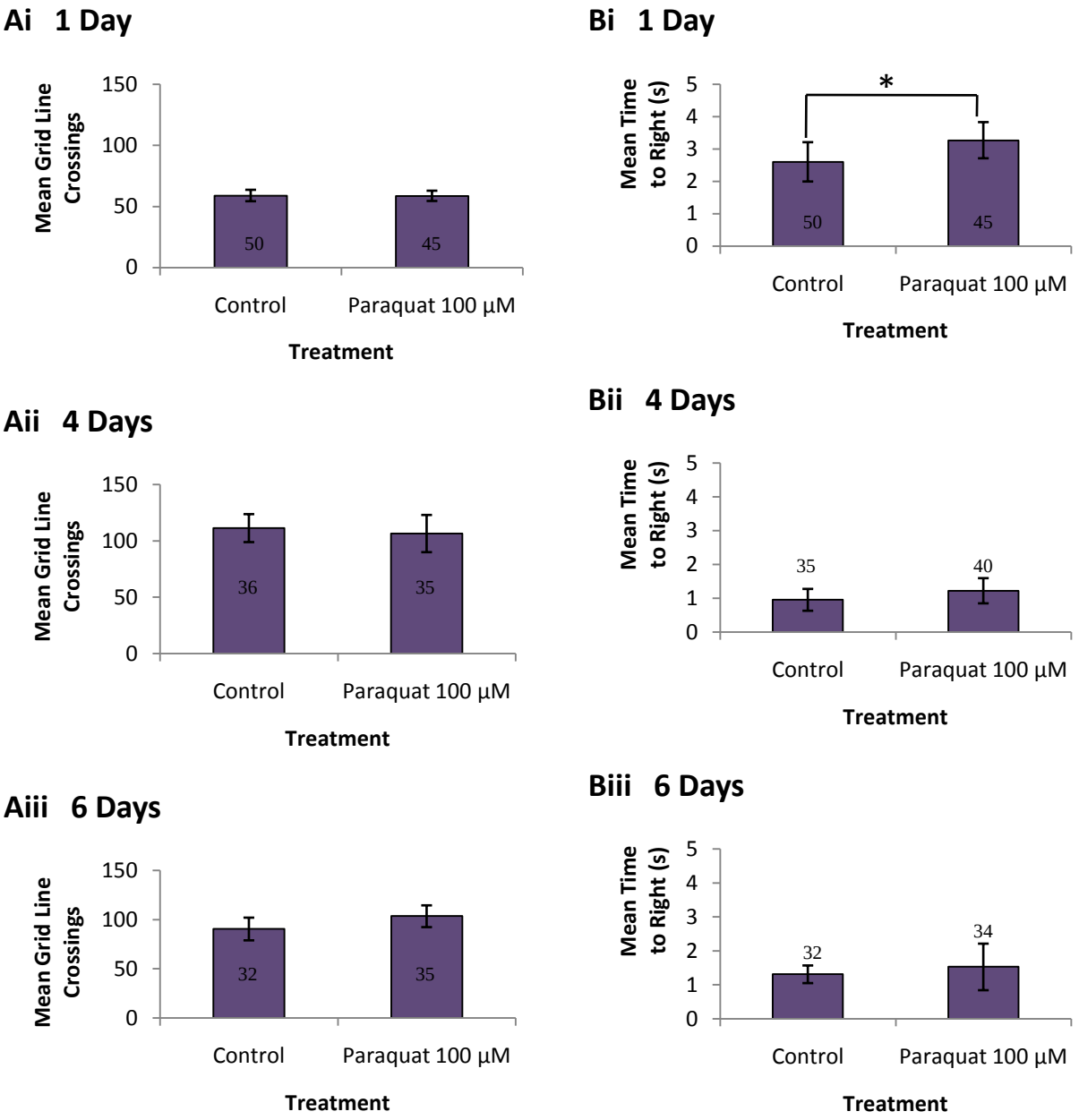

Figure 12. Numbers of bees tested in each group are shown in or above columns. **A.** Activity levels. Mean number of times (±S.E.) one-, four, and six-day old control and paraquat-treated bees crossed grid lines over a two-minute period during the grid line test. Statistical significance was determined by pairwise Mann-Whitney U test. **Ai.** 1 day ($p=0.6845$). **Aii.** 4 days ($p=0.6047$). **Aiii.** 6 days ($p=0.4181$). **B.** Righting behaviour. Mean time taken in seconds (±S.E.) for one-, four- and six-day old control and paraquat-treated bees to right themselves after being flipped onto their backs. The asterisk indicates a significant difference between the groups. Statistical significance was determined by pairwise Mann-Whitney U test. **Bi.** 1 day ($p<0.05$). **Bii.** 4 days ($p=0.2530$). **Biii.** 6 days ($p=0.1328$).

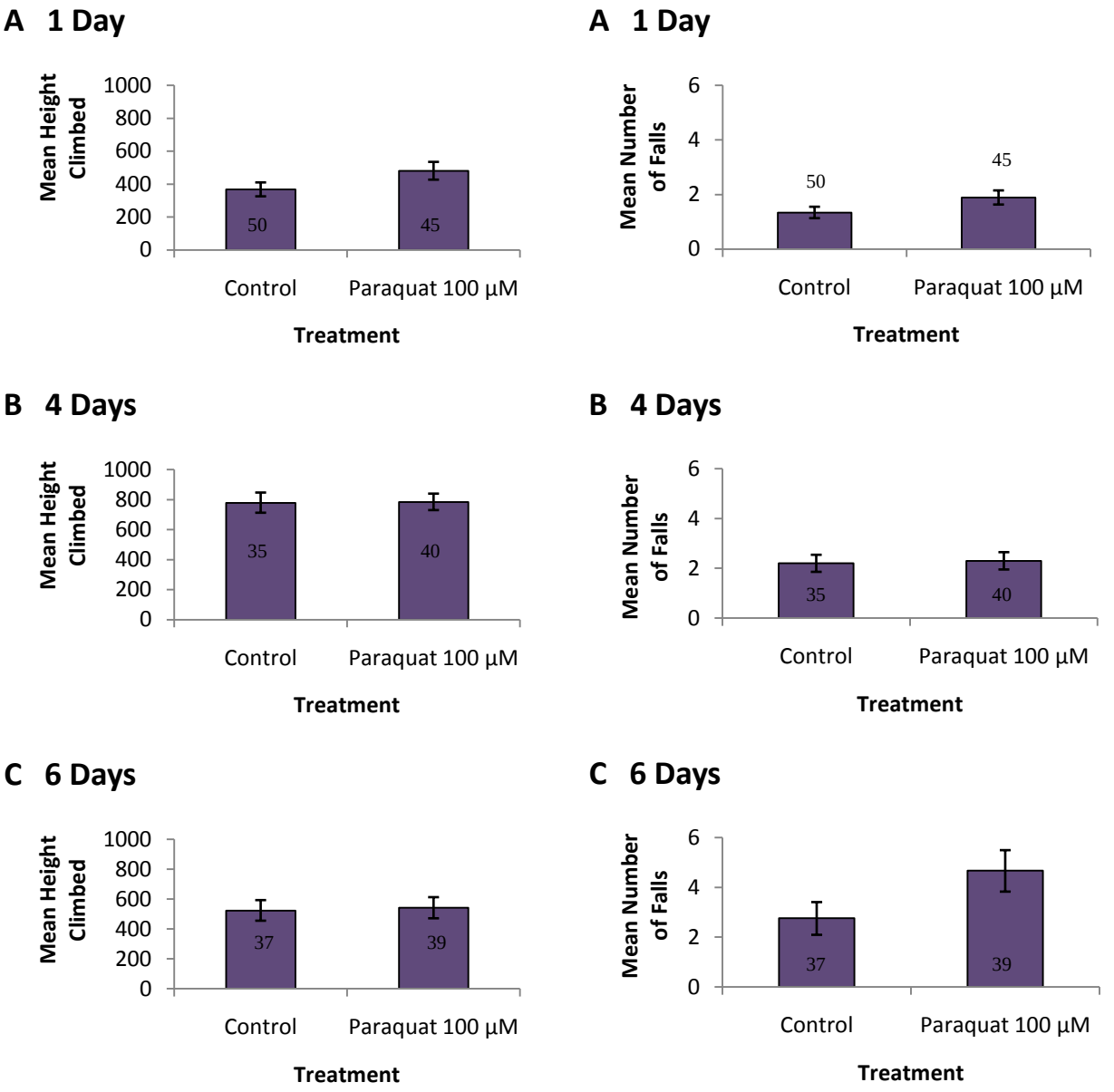

Figure 13. Numbers of bees tested in each group are shown in or above columns. **A.** Climbing ability. Mean height climbed (±S.E.) by one-, four- and six-day old control and paraquat-treated bees over a one-minute period during the climbing test. Statistical significance was determined by pairwise Mann-Whitney U test. **Ai.** 1 day (p=0.1461). **Aii.** 4 days (p=0.5635). **Aiii.** 6 days (p=0.9014). **B.** Falls. Mean number of times one-, four- and six-day old control and paraquat-treated bees fell off the wall of the climbing cylinder (±S.E.) over a one-minute period. Statistical significance was determined by pairwise Mann-Whitney U test. **Bi.** 1 day (p=0.1008). **Bii.** 4 days (p=0.9914). **Biii.** 6 days (p=0.0958).

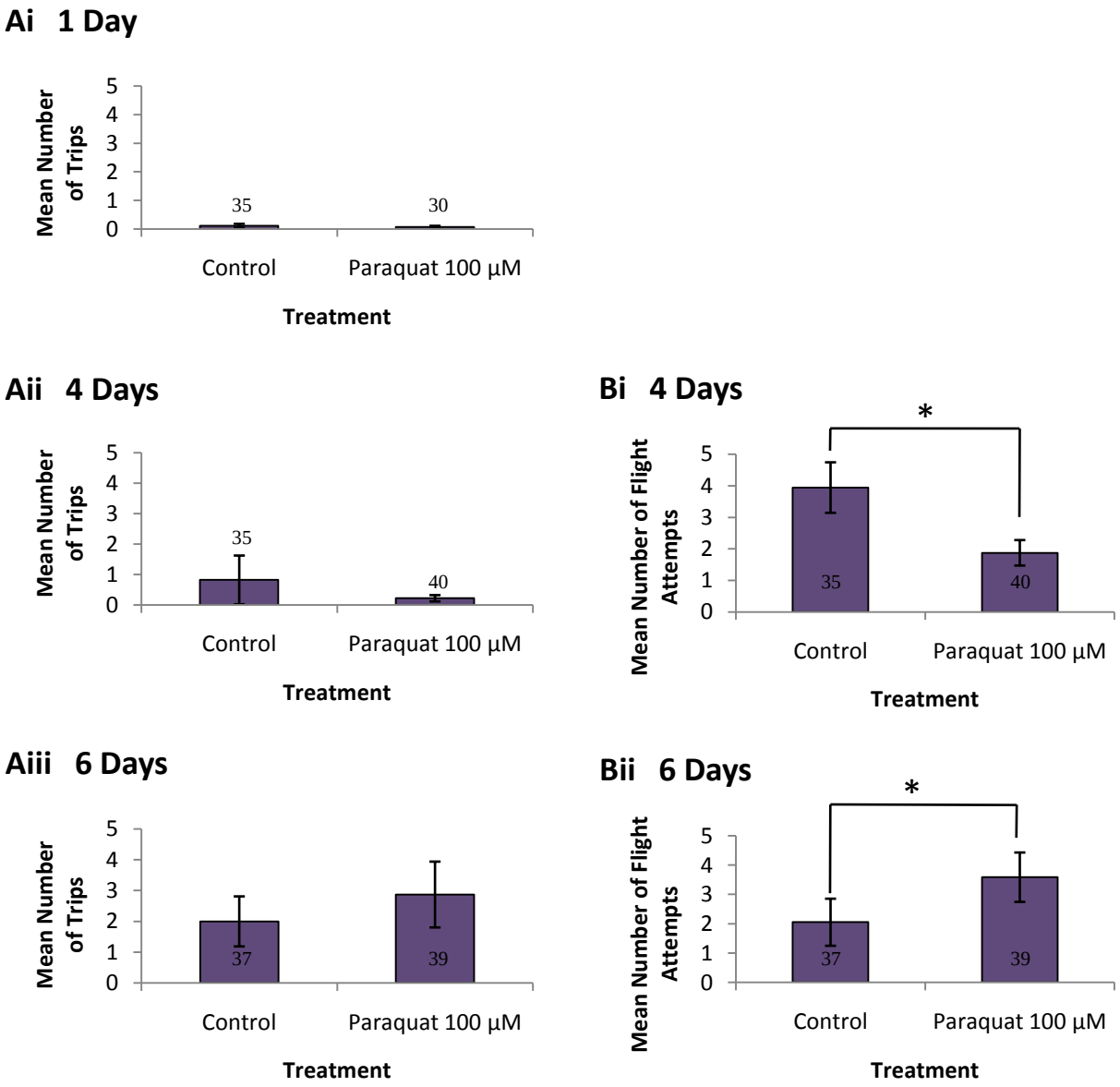

Figure 14. Numbers of bees tested in each group are shown in or above columns. **A.** Trips. Mean number of trips (±S.E.) made by one-, four- and six-day old control and paraquat-treated bees over a one-minute period during the climbing test. A trip was defined as an unsuccessful attempt made by a honey bee to climb onto the wall of the climbing cylinder where it slipped off to one side with at least the back pair of legs still on the floor of the cylinder. Statistical significance was determined by pairwise Mann-Whitney U test. **Ai.** 1 day ($p=0.7648$). **Aii.** 4 days ($p=0.3330$). **Aiii.** 6 days ($p=0.3128$). **B.** Flight attempts. Mean number of flight attempts (±S.E.) made by four- and six-day old control and paraquat-treated bees over a one-minute period during the climbing test. Data from one-day old bees has not been shown as all values were equal to zero. Asterisks indicate significant differences between the groups. Statistical significance was determined by pairwise Mann-Whitney U test. **Bi.** 4 days ($p<0.05$). **Bii.** 6 days ($p<0.05$).

3.2 Age Dependent Changes in Activity Levels

From the results described above it was apparent that although BSSG and paraquat had very little effect on motor activity, activity levels appeared to change significantly with age in bees treated with BSSG and paraquat, as well as in the control group. These age-related changes are examined more closely below (Figures 15-17). The trends were the same in all treatment conditions, therefore the groups were pooled to make a much larger data set.

In all of the behavioural assays used, bees that were one-day old behaved differently than four- and six-day old bees. The behavioural assay used to measure activity levels revealed that both four- and six-day old bees were more active than one-day old bees ($p<0.001$; $p<0.001$) (Figure 15a). One-day old bees also took longer to right themselves when flipped onto their backs than either four- or six-day old bees ($p<0.001$; $p<0.001$) (Figure 15b). There were no differences in activity levels or righting behaviour between four- and six-day old bees (Figure 15).

In a test of climbing ability, both four- and six-day old bees climbed higher than one-day old bees ($p<0.001$; $p<0.001$) (Figure 16a). Four-day old bees also climbed significantly higher than six-day old bees ($p<0.001$). Four- and six-day old bees fell off the wall of the climbing cylinder significantly more often than one-day old bees ($p<0.001$; $p<0.001$) (Figure 16b).

Six-day old bees appeared to have more trouble getting onto the wall of the climbing cylinder than one- ($p<0.001$) and four-day old bees ($p<0.001$), as they made more trips (Figure 17a). One-day old bees did not fly at all, which is a significant deviation from the behaviour apparent in four- and six-day old bees (Figure 17b).

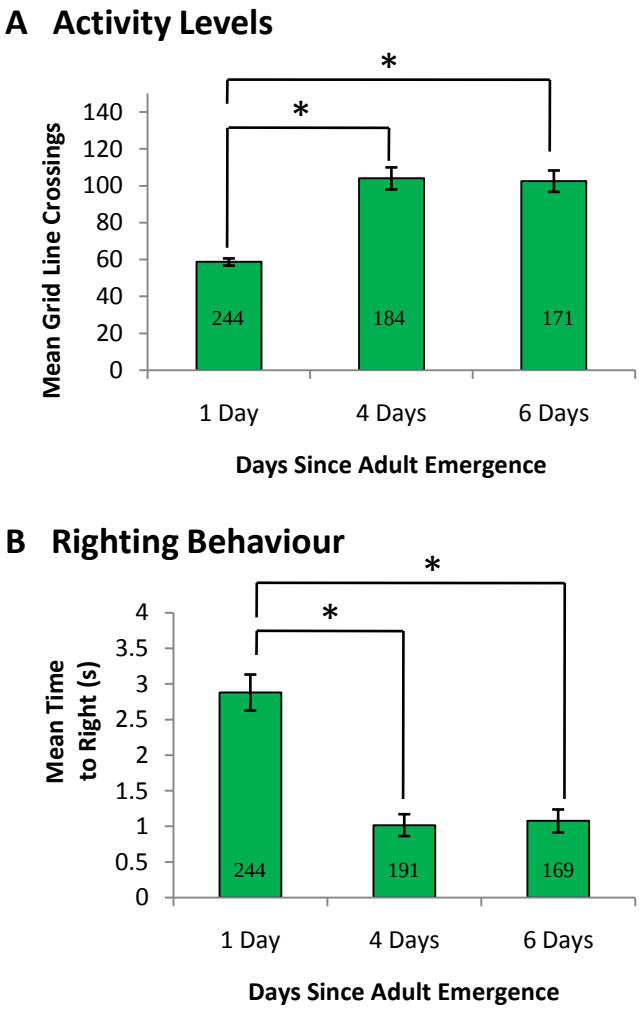

Figure 15. Numbers of bees tested in each group are shown in columns. **A.** Activity levels. Mean number of times one-, four- and six-day old bees crossed grid lines over a two-minute period during the grid line test. Error bars are ± standard error. Asterisks indicate significant differences between the groups. Statistical significance was determined by Kruskal-Wallis test followed by pairwise Mann-Whitney U tests for post-hoc comparisons ($p<0.001$: 1 vs 4 days $p<0.001$; 1 vs 6 days $p<0.001$; 4 vs 6 days $p=0.9620$). **B.** Righting behaviour. Mean time taken for one-, four- and six-day old bees to right themselves after being flipped onto their backs. Error bars are ± standard error. Time was measured in seconds. Asterisks indicate significant differences between the groups. Statistical significance was determined by Kruskal-Wallis test followed by pairwise Mann-Whitney U tests for post-hoc comparisons ($p<0.001$: 1 vs 4 days $p<0.001$; 1 vs 6 days $p<0.001$; 4 vs 6 days $p=0.8607$).

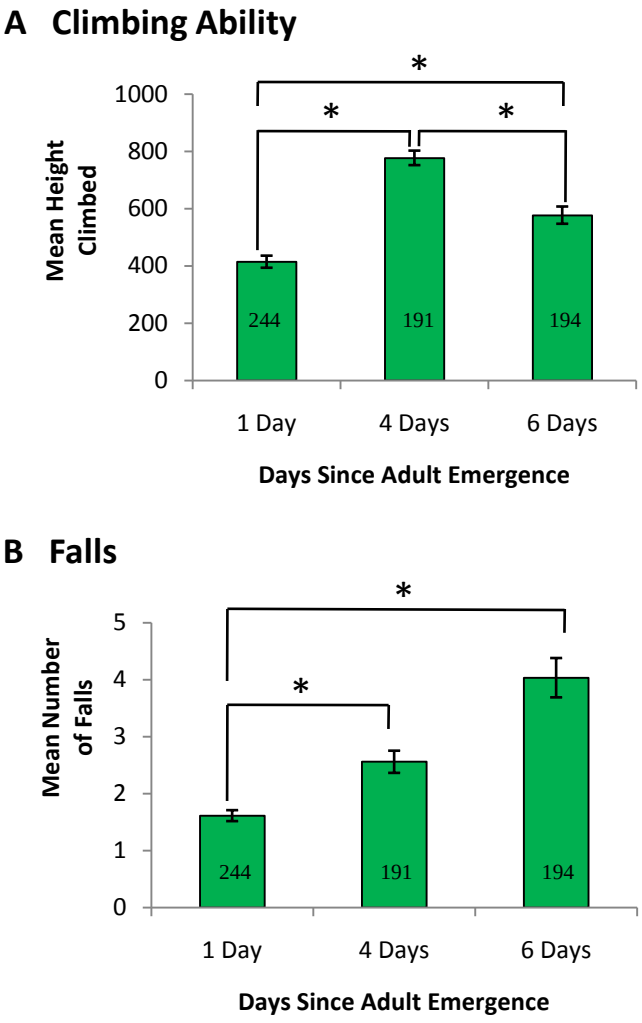

Figure 16. Numbers of bees tested in each group are shown in columns. **A.** Climbing ability. Mean height climbed by one-, four- and six-day old bees over a one-minute period during the climbing test. Error bars are ± standard error. Asterisks indicate significant differences between the groups. Statistical significance was determined by Kruskal-Wallis test followed by pairwise Mann-Whitney U tests for post-hoc comparisons ($p<0.001$: 1 vs 4 days $p<0.001$; 1 vs 6 days $p<0.001$; 4 vs 6 days $p<0.001$). **B.** Falls. Mean number of times one-, four- and six-day old bees fell off the wall of the climbing cylinder over a one-minute period. Error bars are ± standard error. Asterisks indicate significant differences between the groups. Statistical significance was determined by Kruskal-Wallis test followed by pairwise Mann-Whitney U tests for post-hoc comparisons ($p<0.001$: 1 vs 4 days $p<0.001$; 1 vs 6 days $p<0.001$; 4 vs 6 days $p=0.0973$).

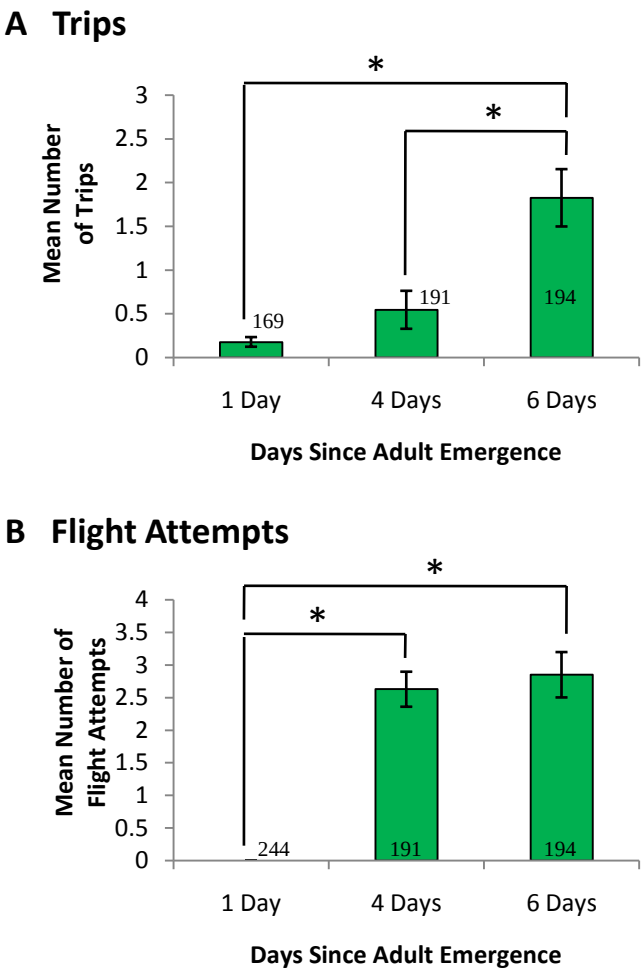

Figure 17. Numbers of bees tested in each group are shown in or above columns. **A.** Trips. Mean number of trips made by one-, four- and six-day old bees over a one-minute period during the climbing test. A trip was defined as an unsuccessful attempt made by a honey bee to climb onto the wall of the climbing cylinder where it slipped off to one side with at least the back pair of legs still on the floor of the cylinder. Error bars are ± standard error. Asterisks indicate significant differences between the groups. Statistical significance was determined by Kruskal-Wallis test followed by pairwise Mann-Whitney U tests for post-hoc comparisons ($p<0.001$: 1 vs 4 days $p=0.9386$; 1 vs 6 days $p<0.001$; 4 vs 6 days $p<0.001$). **B.** Flight attempts. Mean number of flight attempts made by one-, four- and six-day old bees over a one-minute period during the climbing test. Error bars are ± standard error. Asterisks indicate significant differences between the groups. Statistical significance was determined by Kruskal-Wallis tests for overall and post-hoc comparisons ($p<0.001$: 1 vs 4 days $p<0.001$; 1 vs 6 days $p<0.001$; 4 vs 6 days $p=0.062$).

3.3 Effects of β-Sitosterol-β-D-Glucoside (BSSG) and Paraquat on Sucrose Sensitivity

Sucrose sensitivity was measured in seven-day old honey bees by presenting them with water and increasing concentrations of sucrose solution ranging from 0.1% sucrose to 55% sucrose. There was a significant increase in the level of responses as sucrose concentration increased for bees in the control ($p<0.01$) and BSSG 2 μM ($p<0.01$) groups (Figure 18a). This was not due to sensitisation as there were no significant differences in responses to water as sucrose concentration increased in any of the treatment groups (Figure 18b).

Sucrose response threshold was measured in all groups by examining responses to sucrose and water and determining the concentration of sucrose at which responses were significantly greater than initial responses to water. The sucrose response threshold was a concentration of 30% sucrose for bees in control ($p<0.05$) and BSSG 2 μM ($p<0.01$) groups, and 55% sucrose for bees in BSSG 20 nM ($p<0.05$) and BSSG 200 μM ($p<0.05$) groups (Figure 18c). Bees treated with paraquat never reached a threshold of responding to sucrose that was significantly greater than responses to water.

Kruskal-Wallis tests were used to compare treatment groups at 30% and 55% sucrose as these were equal to or higher than the sucrose response thresholds for most of the treatment groups. There was a significant difference between the treatment groups at 55% ($p<0.01$), but not at 30% sucrose. Mann-Whitney U tests comparing treatment groups to controls at a concentration of 55% sucrose showed that responses were affected in both the BSSG 200 μM ($p<0.01$) and paraquat 100 μM ($p<0.001$) groups (Figure 18a).

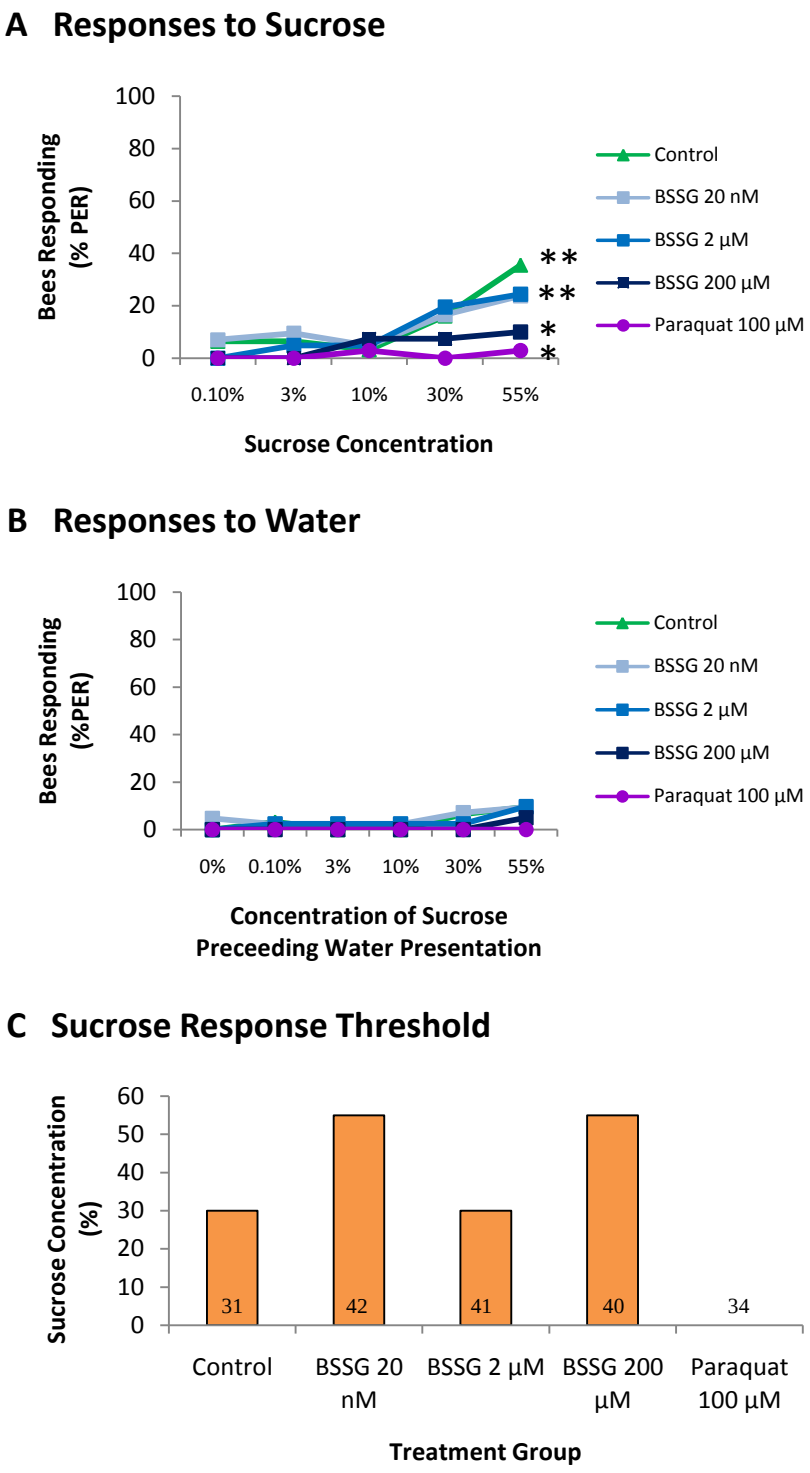

Figure 18. Numbers of bees tested in each group are shown in columns in graph C. **A.** Responses to sucrose. Percentage of control, BSSG- and paraquat-treated bees responding to sucrose solutions at concentrations of 0.1%, 3%, 10%, 30% and 55% held near one antenna. Two asterisks together indicate significantly greater responding as sucrose concentration increases within a treatment group. One asterisk

Figure 18a also indicates that when a sucrose droplet is held close to one antenna, as opposed to the antennae being stimulated by direct touching of sucrose, seven-day old bees reared in cages do not exhibit a high level of responding to low, and even moderate, concentrations of sucrose solution; at 10% sucrose solution less than 10% of bees responded, and with a 30% solution the number of bees responding was fewer than 20%.

3.4 Effects of β-Sitosterol-β-D-Glucoside (BSSG) and Paraquat on Olfactory Learning and Memory

The effect of BSSG and paraquat on learning ability in eight-day old honey bees was tested using single trial olfactory conditioning. Treatment groups were compared with the control group using Chi-square analysis and the critical p-value was adjusted to ensure that overall significance

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next to a data point indicates it is significantly different from the control at the same sucrose concentration. Statistical significance was determined by Kruskal-Wallis test followed by Mann-Whitney U tests as post-hoc comparisons where appropriate (control $p<0.01$; BSSG 20 nM $p=0.053$; BSSG 2 µM $p<0.01$; BSSG 200 µM $p=0.119$; paraquat 100 µM $p=0.555$. 30% sucrose $p=0.067$; 55% sucrose $p<0.01$: control vs BSSG 20nM $p=0.2826$; control vs BSSG 2 µM $p=0.3120$; control vs BSSG 200 µM $p<0.01$; control vs paraquat 100 µM $p<0.001$). **B.** Responses to water. Percentage of control, BSSG- and paraquat-treated bees responding to water held near one antenna after each sucrose presentation. Statistical significance was determined by Kruskal-Wallis test (control $p=0.144$; BSSG 20 nM $p=0.523$; BSSG 2 µM $p=0.206$; BSSG 200 µM $p=0.074$; paraquat 100 µM $p=1.000$). **C.** Sucrose response threshold. The threshold at which responses to sucrose are significantly greater than responses to water. Statistical significance was determined by Kruskal-Wallis test (control 0.1% $p=0.154$; 3% $p=0.154$; 10% $p=0.317$; 30% $p<0.05$; 55% $p<0.001$; BSSG 20 nM 0.1% $p=0.647$; 3% $p=0.400$; 10% $p=1.000$; 30% $p=0.080$; 55% $p<0.05$; BSSG 2 µM 0.1% $p=1.000$; 3% $p=0.155$; 10% $p=0.155$; 30% $p<0.01$; 55% $p<0.01$; BSSG 200 µM 0.1% $p=1.000$; 3% $p=1.000$; 10% $p=0.079$; 30% $p=0.079$; 55% $p<0.05$; paraquat 100 µM 0.1% $p=1.000$; 3% $p=1.000$; 10% $p=0.317$; 30% $p=1.000$; 55% $p=0.317$).

was maintained at the 0.05 level. Neither BSSG, at any of the treatment concentrations, nor paraquat, affected the percentage of bees responding to a conditioned olfactory stimulus after a single conditioning trial relative to the control group. This was true following olfactory conditioning of honey bees using either 10% sucrose (Figure 19a) or 55% sucrose (Figure 19b) as the unconditioned stimulus.

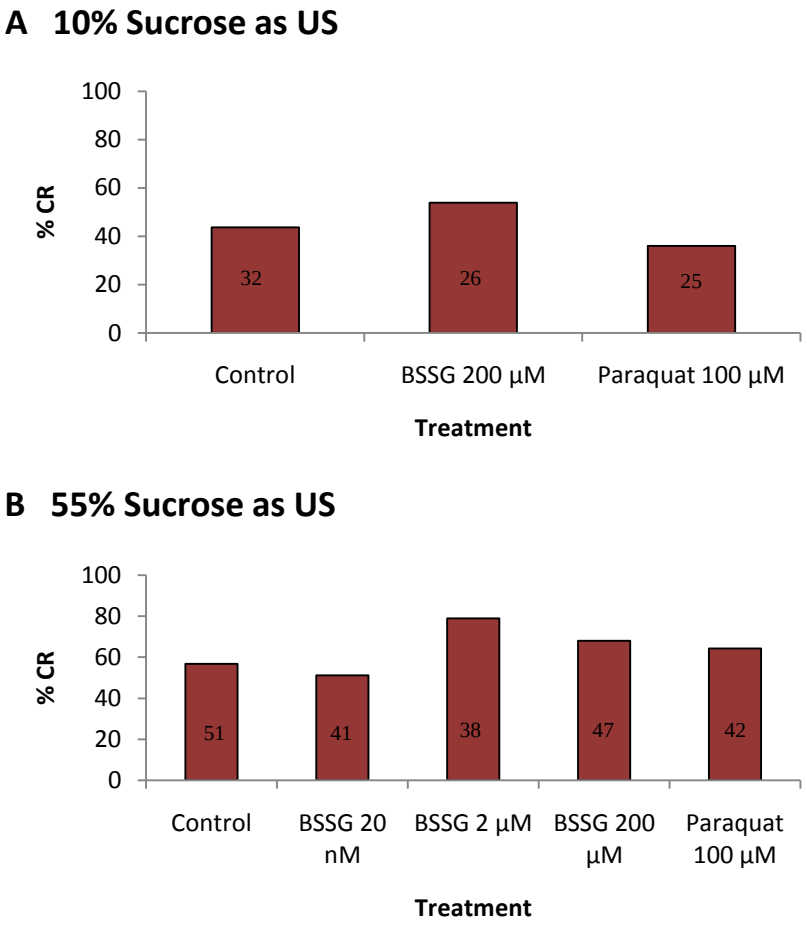

Figure 19. Percentage of control, BSSG- and paraquat-treated bees responding to a conditioned olfactory stimulus with either 10% or 55% sucrose solution as the unconditioned stimulus during conditioning. Conditioned responses were tested one hour after a single conditioning trial. Statistical significance was determined by Chi-square analysis comparing treatment condition

with control. Numbers of bees tested in each group are shown in columns. **A.** 10% sucrose solution (control vs BSSG 200 μM p=0.444; control vs paraquat 100 μM p=0.554). **B.** 55% sucrose solution (control vs BSSG 20 nM p=0.589; control vs BSSG 2 μM p=0.029 [adjusted critical p=0.0125]; control vs BSSG 200 μM p=0.252; control vs paraquat 100 μM p=0.467).

4. DISCUSSION

4.1 Age Dependent Changes in Activity Levels

The results of this study show marked changes in the motor behaviour of worker bees during the first week of adult life. One-day old bees were less active than older bees, they were slower at righting themselves, and their climbing ability was poor. Their performance in climbing was probably not due to lower activity levels or differences in the speed of the bees as there was more than enough time allowed during the test for bees of any of the ages to climb to the top of the cylinder several times. Therefore, either older bees were more motivated to climb higher, or they were simply better at it. The additional finding that four-day old bees were better at climbing than six-day old bees suggests a motivational component to climbing ability in worker bees. The fewer number of trips and falls in very young bees suggests that their coordination and balance was better than in older bees. Furthermore, one-day old bees did not fly at all. This is consistent with the findings of Roberts and Elekonich (2005) who report that flying in honey bees develops within the first three to four days after emergence and that two-day old bees are able to hover.

4.2 Motor Control was not Affected by β-Sitosterol-β-D-Glucoside (BSSG) or by Paraquat

Perhaps surprisingly, BSSG did not have any effect on activity levels in bees regardless of the age of the bees, or the concentration of BSSG administered. Interestingly, there was a difference

in righting behaviour at six days of age: control bees took longer to right themselves than bees treated with BSSG at a concentration of 20 nM. However, there was no evidence of improved righting ability at the other two BSSG concentrations tested. This effect of greater speed during righting may be something that is seen only with low concentrations of BSSG. Varying effects due to differing concentrations have been seen with alcohol. Locomotor activity in the fruit fly, *Drosophila melanogaster*, is initially increased with exposure to alcohol, and after a period of time activity decreases again. However, at high concentrations the increase in activity is rapid and brief, followed by an equally rapid reduction; at low concentrations of alcohol the activity increase is of longer duration and the reduction slower (Parr et al., 2001).

One possible reason why BSSG administration did not lead to motor impairments in the present study may have been because healthy neurons have the ability to compensate for damaged ones. In humans with age-related neurological disorders, clinical symptoms may be apparent only after substantial damage has occurred in the brain, as healthy neurons have the ability to compensate for damaged or dead neurons until a threshold of neuron loss is reached, at which point the behavioural and motor deficits become observable (Shaw & Wilson, 2003). The neural mechanisms controlling locomotion and movement are similar in insects and mammals (Kien and Altman, 1992), and previous studies have found significant effects from treating mice with BSSG (Khabazian et al., 2002; Wilson et al., 2002; Shaw & Wilson, 2003; Wilson et al., 2003; Wilson et al., 2004; Wilson & Shaw, 2006). If the toxins in the present study had been administered at higher doses or over a greater length of time then motor symptoms may have become apparent.

The lack of impairment in motor behaviour resulting from BSSG treatment might also be attributable to the way the toxins were delivered to the bees. The toxin treatments were

administered in food and it is not known how much pollen was ingested by the bees. If the honey bees in the present study actively avoided food laced with toxin, levels ingested by the bees may have been lower than expected. The results obtained with paraquat support this possibility. Although paraquat did have an effect on righting behaviour and flying, the effects were less dramatic than expected and suggest that feeding bees toxins may not be an ideal way to test toxicity. Paraquat is a widely used herbicide that has toxic effects in many species (Brooks et al., 1999; Di Monte, 2001; Girardot et al., 2004; Uversky, 2004; Krishnan et al., 2007). Paraquat injected into adult firebugs, *Pyrrhocoris apterus* leads to changes in the brain, altered blood protein profile, reduced fertility in females and a reduced life span (Krishnan et al., 2007). Paraquat also triggers stress response in gene transcription in adult *Drosophila melanogaster* (Girardot et al., 2004). Exposure to high levels of paraquat can affect the lungs, liver, kidneys, and brain in mammals and can lead to death when eaten (Brown et al., 1996; Uversky, 2004). Paraquat exposure has also been linked to Parkinson's disease (Shimizu et al., 2003) and has been shown to increase α-synuclein in the substantia nigra and frontal cortex in mice (Manning-Bog et al., 2002). α-Synuclein is a component of Lewy bodies, or intraneuronal inclusions, which are seen in the pathology of Parkinson's disease (Di Monte, 2001; Manning-Bog et al., 2002; Uversky, 2004). MPP^+, a metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), has a chemical structure similar to paraquat (Di Monte, 2001), and MPTP is a synthetic heroin which has been shown to kill dopaminergic neurons in the substantia nigra and induce a type of Parkinson's Disease (Thiruchelvam et al., 2000b; Di Monte, 2001). Uversky (2004) states that it takes more than 80% of dopaminergic neurons to die before symptoms of Parkinson's disease are seen.

In mice, paraquat administration leads to a dose-dependent reduction in dopaminergic neurons in the substantia nigra and a reduction in dopaminergic nerve terminals in the striatum (Brooks et al., 1999). The nigrostriatal pathway is involved in regulating movement and dopamine is a central nervous system neurotransmitter for neurons involved in this pathway and others (Uversky, 2004). Therefore, changes in dopamine levels should be monitored in any future studies using honey bees. Low levels of paraquat can increase the susceptibility of dopaminergic neurons and the nigrostriatal system to damage by other toxins. At relatively high doses paraquat causes a reduction in dopaminergic neurons, but when paired with MPP^+, significantly greater cell death is seen with much lower doses of both chemicals; no reduction in dopaminergic neurons is seen when either paraquat or MPP^+ is used exclusively at the same doses (Shimizu et al., 2003). Mice injected with paraquat along with a fungicide, maneb, exhibit an increase in dopamine activity combined with a reduction in locomotor activity after injections of both together but not either alone (Thiruchelvam et al., 2000a; Thiruchelvam et al., 2000b). Furthermore, MPTP decreased locomotor activity in mice that had previously been given paraquat but had no effect on control mice. Paraquat effects appear to be age-dependent, as young animals show the greatest deficits and the least recovery, (Uversky, 2004). It has been suggested that if the nigrostriatal dopamine system is exposed to a combination of environmental or agricultural neurotoxins during development this can make it more vulnerable to later chemical exposures that would not normally be as detrimental without the previous exposure (Thiruchelvam et al., 2000a; Uversky, 2004). In the present study bees were exposed to either BSSG or paraquat, but not both together. It is possible that BSSG may have had an effect on honey bee motor behaviour if it had been paired with paraquat. Future research should examine this idea by pairing the two toxins and/or administering paraquat earlier during development followed later by BSSG.

The concentration of paraquat used in this study was 100 µM, whereas previous studies with *Drosophila melanogaster* and *Pyrrhocoris apterus* have fed paraquat at concentrations of 5 mM and 15 mM (Zou et al., 2000; Girardot et al., 2004), or have injected paraquat directly into the body (Krishnan et al., 2007). This suggests that a higher dose of paraquat, a longer length of exposure, and/or a different administration method than was used in this study may elicit further effects in honey bees. Future studies should examine neuronal death after administration of paraquat to bees, as well as using longer exposures at a variety of doses, administering paraquat by injection into the haemolymph as an alternative to feeding, and using larvae or pupae instead of adult bees. Pairing paraquat with another chemical may also produce greater effects than using paraquat alone. The next step is to then examine neurotransmitter pathways, especially changes in dopamine levels, along with motor neuron density to determine how these are affected by paraquat.

4.3 β-Sitosterol-β-D-Glucoside (BSSG) Reduces Sucrose Responsiveness in Bees

Despite the lack of effect of BSSG and paraquat on the performance of motor behaviours, sucrose responsiveness was affected by both toxins. At concentrations of 20 nM and 200 µM, BSSG reduced sucrose responsiveness in young worker bees and raised the sucrose response threshold (Figure 18). Honey bees treated with 100 µM paraquat also showed impaired responses to sucrose compared to control bees, and were unable to detect a difference between water and sucrose at any concentration. In paraquat-treated bees, the number of bees responding to sucrose was fewer than 5% across all concentrations of sucrose tested. The results suggest, however, that

bees could still detect sucrose if it was physically applied to the antennae, as they responded to sucrose and to odours during olfactory conditioning.

Honey bee antennae are multi-sensory organs that contain olfactory, taste, humidity, temperature and mechanical receptors (Winston, 1987; Resch et al., 1998; Haupt, 2004; Farooqui, 2007; de Brito Sanchez et al., 2007). The base section of the antennae is the scape, which is attached to the head of the honey bee and linked to the flagellum by the pedicel. The sensory region in each honey bee antennae is the flagellum, which in workers is divided into ten segments that house the sensilla that contain the sensory receptor cells. The main olfactory sensilla on the antennae are called pore plates and they are located on the eight most distal segments of the flagellum. There are approximately three thousand pore plates on a single antenna of a worker bee. Odour molecules pass through rows of fine pores and are transported to receptor cells beneath the plate (reviewed in Winston, 1987). Gustatory receptors are situated inside sensillae located on the tips of the antennae (Haupt, 2004; de Brito Sanchez et al., 2005). If sucrose is touched to the antennae, taste sensilla are able to respond to sucrose at a concentration of 0.1% (Haupt, 2004). Humidity receptors are located also on the second outermost segment of the antennae (Resch et al., 1998) and these may have contributed to the bees' responses to droplets of sucrose held close to the antennae.

In honey bees, as in mammals, olfactory ability declines with age, something that is commonly seen in aging humans (Farooqui, 2007). Olfactory deficits are a common symptom of age-related neurological disease in humans (Ahlskog et al., 1998; Tissingh et al., 2001; Farooqui, 2007). Patients with Parkinson's disease show deficits in odour detection, discrimination and identification, the severity of which is correlated with the progression of the disease (Tissingh et

al., 2001). Olfactory deficits are also seen in parkinsonism-dementia complex (PDC), amyotrophic lateral sclerosis (ALS), parkinsonism, and dementia in Guam, where there is a higher incidence of these neurological diseases than elsewhere in the world (Ahlskog et al., 1998). Olfactory impairments associated with age-related neurological disease are first seen early in the disease progression (Tissingh et al., 2001). Tissingh et al. (2001) found that undiagnosed and untreated patients with mild symptoms of Parkinson's disease also showed olfactory deficits, and they suggested that olfactory deficits are present before the onset of motor dysfunction. In humans, odours are first encountered by the olfactory epithelium in the roof of the nasal cavities inside the nose, and oxidative stress in the olfactory epithelium in patients with Alzheimer's disease leads to olfactory impairment (reviewed in Farooqui, 2007). This suggests that loss of smell in neurological disease may first be happening at the peripheral level rather than in the olfactory centres of the brain. The current study shows that BSSG and paraquat lead to changes in sucrose responsiveness. In honey bees, antennal olfactory receptor neurons appear to be modulated by biogenic amines (Vergoz et al., in press) and it is possible that BSSG and paraquat were also acting at this peripheral level.

An interesting finding in the present study was that seven-day old bees did not respond well to low, and even moderate, concentrations of sucrose solution. Less than 10% of bees responded to a sucrose concentration of 10%, and even with a sucrose concentration of 30% less than 20% of bees responded by extending the proboscis. Sucrose response threshold is determined as the concentration of sucrose at which responses are greater than responses to water alone (Page et al., 1998). If a bee responds to low concentrations of sucrose then it has a low sucrose response threshold and therefore a high sensitivity to sucrose. How bees respond to sucrose can serve as an estimate of their behavioural state (Scheiner et al., 2003), as the way bees respond to sucrose

differs according to factors such as genotype, satiation, treatment with pheromones and hormones, and whether they are pollen or nectar foragers (Page et al., 1998; Pankiw & Page, 1999; Scheiner et al., 2001a; Scheiner et al., 2001b; Pankiw et al., 2001; Pankiw & Page, 2003). Sucrose responsiveness is also correlated with walking activity in newly emerged honey bees (Humphries et al., 2005). Differences between high- and low-pollen-hoarding strains of bees are apparent at zero to two days of age, with low-strain bees having much higher response thresholds than high-strain bees (Pankiw & Page, 1999). High-strain bees are more responsive to water and low sucrose concentrations than low-strain bees, both before and after they have started foraging (Scheiner et al, 2001b). Bees that are highly sensitive to sucrose learn faster on tactile and olfactory learning paradigms, regardless of genotype (Scheiner et al., 2001a). Individual honey bees can change their sucrose response thresholds on the basis of experience and perception of sugar. Bees that have previously foraged on high concentrations of sucrose are less likely to respond to lower concentrations of sucrose than bees previously collecting low concentrations, although high-strain bees are more responsive to sucrose than low-strain bees regardless of the sucrose concentration they have previously been collecting (Pankiw et al., 2001). Perception of sugar in honey bees may also be dependent on satiation level, as bees that have been fed are less responsive to both sucrose and water than hungry bees (Page et al., 1998; Pankiw et al., 2001). Changes in the pheromones and hormones that honey bees are exposed to can also affect sucrose-response thresholds. Bees raised in the presence of queen mandibular pheromone (QMP) have a higher response threshold than bees raised without QMP, and brood pheromone (BP) raises the sucrose-response threshold of honey bee foragers (Pankiw & Page, 2003). Pollen foragers have lower sucrose-response thresholds than nectar foragers (Page et al., 1998; Pankiw et al., 2001). Sucrose responsiveness also increases as bees age, with middle-aged bees responding to lower concentrations of sucrose than young bees (Goode et al., 2006).

The honey bees used in the present study were all taken from the same colony and the genetic make-up in relation to the pollen-hoarding gene or the proportion of pollen to nectar foragers within the colony is not known. However, the bees tested were all relatively young, which may in part explain why they did not respond to low concentrations of sucrose. The high sucrose response thresholds seen in the present study may also be attributable to satiation level, as the bees were not necessarily hungry. The honey bees were allowed to feed ad libitum across the experiment from a constant food source, and so the satiation level of the bees was not known. If they were satiated they may have lacked the motivation to respond to lower concentrations of sucrose.

4.4 β-Sitosterol-β-D-Glucoside (BSSG) has no Affect on Associative Olfactory Learning

Neither BSSG nor paraquat had an effect on single trial olfactory conditioning in worker honey bees. This too was surprising, as previous studies have shown that honey bees fed the insecticides and pesticides endosulfan, baytroid, sevin, fipronil, deltamethrin and prochloraz are impaired on olfactory learning tests (Abramson et al., 1999; Decourtye et al., 2005), and learning performance is also diminished when honey bees are fed ethanol prior to conditioning (Mustard et al., 2008). Mice fed cycad flour show a reduction in cognitive function correlated with neuron loss (Schulz et al., 2003; Shaw & Wilson, 2003; Wilson et al., 2002; Wilson et al., 2004; Wilson & Shaw, 2006). Learning may have contributed to the lack of effects of BSSG and paraquat in the present study. Conditioned taste aversion to food has been shown in rats injected with paraquat immediately after eating, at concentrations that do not elicit obvious signs of toxicity

(Edmonds & Edwards, 1996). The reduction in sucrose sensitivity in paraquat and BSSG 20 nM and 200 μM groups in the present study demonstrates that the bees must have ingested some BSSG and paraquat, but it is possible the toxin treatment generated food aversion in the bees as toxin-laced food was available throughout the experiments. Previous studies involving paraquat have used both ingestion and injection as administration methods (Edmonds & Edwards, 1996; Brooks et al., 1999; Thiruchelvam et al., 2000a; Thiruchelvam et al., 2000b; Zou et al., 2000; Girardot et al., 2004; Krishnan et al., 2007). Future studies involving honey bees should administer toxins by injection directly into the haemolymph, and also try administering BSSG to larvae and pupae to see how this affects their development, as fungicides and pesticides are more toxic to larvae and pupae than to adult bees (Mussen et al., 2004).

The present study demonstrates that learning in young bees can be influenced by the concentration of sucrose solution used as the reward. Learning levels of the bees were between 30% and 60% with the moderate concentration of sucrose solution (10%), and between 50% and 80% of the bees showed successful PER conditioning when the high concentration of sucrose solution (55%) was used. The levels of learning seen in the present study may have also been affected by the age of the bees, as the bees used were eight-days post-emergence. Learning in honey bees can be affected by the age of the bees at the time of testing. Previous studies have shown that fifteen-day old honey bees and adult foragers exhibit levels of learning at 80-85% during single-trial olfactory conditioning (Morgan et al., 1998; Ray and Ferneyhough, 1999). Newly emerged bees show very little ability with olfactory conditioning, with levels of learning increasing during the first week after emergence (Morgan et al., 1998), and a decline in learning acquisition in aging honey bees may be due more to the social role of the bee than its age (Behrends et al., 2007). Maleszka and Helliwell (2001) showed that olfactory conditioning using

a single trial did not induce learning until bees were five to six days old, and only reached adult levels when the bees reached eight days of age. When reared in an artificial environment, levels of olfactory conditioning in young honey bees is not comparable to that of forager bees until they are ten-days old, and although five- and six-day old bees demonstrate PER conditioning and retention, it takes several trials before a high level of responding is seen (Ray and Ferneyhough, 1997). Arenas and Farina (2008) argued that honey bees could learn to associate an odour with a food reward at five to six days after emergence, as nearly 80% of six-day old bees in their study exhibited olfactory conditioning. However, they used a very high concentration of sucrose solution as a reward during conditioning and that may have affected the learning of the bees.

Satiation level is a likely factor to have influenced learning in the current study, as bees were allowed to feed ad libitum throughout the experiment. The bees may have therefore already been satiated before conditioning, and so would have been less able or less motivated to learn the association. The effect of satiation on learning has been largely overlooked in the literature, as studies tend to use hungry bees during conditioning experiments to increase their motivation to learn. When bees are satiated they are less likely to show acquisition of a conditioned response in learning trials, and this is more pronounced for nurse bees than foragers (Ben-Shahar & Robinson, 2001). Friedrich et al. (2004) showed that feeding honey bees four hours before training on an olfactory conditioning paradigm reduced their ability to form short-term memories after one learning trial. Acquisition and memory formation for long-term memory was also affected. The authors concluded that satiation during conditioning impaired memory formation in both single- and multiple-trial learning (Friedrich et al., 2004). The bees in the present study had a low sensitivity to sucrose and this may also have accounted for the low levels of learning

seen here, as honey bees that are highly sensitive to sucrose learn faster during olfactory learning paradigms (Scheiner et al., 2001a).

4.5 Conclusions

The results of this study show that BSSG affects sucrose responsiveness in bees. Whether, with prolonged exposure, or at concentrations higher than those used in the present study, effects on learning and motor activity would become apparent in bees, as in mammals, awaits further investigation. Perhaps in bees, as in mammals, effects on taste and olfaction are the first to become apparent.

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APPENDIX A

Pollen Patty Recipe

(From Giles, 1991)

9.2g	Lactalbumin
18.8g	Brewer's Yeast
52g	Sugar
20g	Pollen
15-20ml	Water

Weigh out sugar and pollen and grind together in a mortar with a pestle to a fine powder. Add the lactalbumin and Brewer's yeast, mix, then add water slowly. Be careful not to add too much water or the patty will be too wet. Shape into patties between grease-proof paper. Makes about four patties.