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Effects of cage volume and bee density on survival and nutrient intake of honeybees (*Apis mellifera* L.) under laboratory conditions

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Abstract

Laboratory experiments are vital to exploring the causes of pollinator loss, but for these experiments to be informative they should attempt to replicate the hive environment and conserve social interactions. It is unclear how honeybee density and group size affect survival and behaviour in the laboratory. We manipulated cage volume (125-1312 ml) and honeybee group size (10-180 bees) and tested the effects on survival and feeding behaviour. Bees were allowed to regulate their intake from two liquid diets with dry ingredient protein: carbohydrate (P:C) ratios of 0:1 and 1:50 (w/w). Intake was consistent across cages, showing that feeding behaviour is largely unaffected by cage conditions. High survival was recorded in cages with a volume of 2.08 ml/bee, which falls within the natural range of 1.9-3.8 ml/bee in nest sites, and in groups of <60 bees. We suggest that cage volume is more important than group size, and that cage dimensions should be adjusted so that each bee has <3.0 ml of space.

Keywords: **cage design / laboratory studies / nutrient regulation/ survival/ honeybee**

1. Introduction

Honeybees are effective pollinators of both wild plants and large scale agricultural crops (vanEngelsdorp et al. 2009; Potts et al. 2010; reviewed by Dicks et al. 2013; VanBergen 2013), which naturally prompts an interest in honeybee health. Researchers are attempting to determine how factors including pesticides (e.g. Chauzat et al. 2009), parasites (e.g.

Le Conte et al. 2010), diseases (e.g. Evans and Schwarz 2011) and nutrition (e.g. Di Pasquale et al. 2013) contribute to these declines. The rapid international spread of honeybee diseases (Human et al. 2011) and parasites such as *Varroa destructor* (Rosenkranz et al. 2010), means that collaborative research projects in different laboratories are vital for understanding threats to honeybee health. During these laboratory studies natural conditions should be replicated as accurately as possible to ensure that data are informative; this requires a thorough understanding of how environmental conditions affect the biology and behaviour of honeybees.

Inside the hive, the high temperature and humidity required for rearing brood are maintained by thermoregulation of worker bees (Oertel 1949; Jones et al. 2004; Human et al. 2006). However, the volume of the nest and its insulating properties will determine the effort required by bees to keep the internal conditions stable and wild colonies make use of this criterion to select nest sites. Wild colonies selected nest sites with volumes of between 30-60 L, from which the average available space per bee under natural conditions is calculated between 1.9 and 3.8 ml/bee by adjusting for swarm size (Seeley and Morse 1976, McNally and Schneider 1996, Vaudo et al. 201,2 McMenamin et al. 2017). Swarms also selected smaller nest sites in apiary conditions (McMenamin et al. 2017). In Kenyan apiaries, Langstroth hives with a volume of 40 L are preferred by migrating swarms over the larger Kenyan top bar hives (52.5 L) and traditional log hives (42 L) (Crane 1994 McMenamin et al. 2017). Under laboratory conditions, the temperature and humidity can be controlled by keeping bees in temperature controlled incubators, while the volume of the nest site can be replicated through the use of specialised hoarding cages to house honeybees in groups (Williams et al. 2013).

Another important environmental factor to consider when studying honeybees is the social interactions in the hive. Bees are eusocial insects and their behaviour relies on social interactions between colony members (Winston and Michener 1977). Social interactions of adult worker honeybees include trophallaxis (feeding each other, reviewed by Crailsheim 1992), the waggle dance directing other bees to forage (von Frisch and Lindauer 1956) and huddling together in groups to thermoregulate (Lindauer 1955). These social interactions shape individual honeybee behaviour, for example, nutrient gathering behaviour of forager bees is influenced by the composition of the trophallactic secretions received from nurse bees (Camazine et al. 1998) or thermoregulation within the brood nest (Basile et al 2008). This means that in a hive social interactions are necessary for bees to regulate colony temperature, find forage and care for young. Therefore, in any experiment that aims to study honeybee behaviour, it is vital that bees are housed in groups so that the social interactions are preserved.

Group size could have a pronounced effect on the behaviour of caged bees (c.f. Hepburn et al 2014). For example, when the effects of toxins are studied in cage experiments, some bees will feed directly on the available diet while others are fed through trophallaxis, as in the hive (Brodschneider et al. 2017). Since bees get exposed to toxic compounds through their diet, this social interaction could lead to an unequal toxic exposure between nest mates, with the bees feeding directly on the diet having a higher exposure (Brodschneider et al. 2017). These authors found that larger test groups of bees distribute food containing toxic compounds more evenly among individual bees, suggesting that group size of caged bees could affect the reliability of pesticide studies.

Group size can further affect survival and the amount of hoarding (Rinderer and Baxter 1978), as well as task allocation and physiological processes like wax secretion and egg laying (Hepburn et al. 2014).

While group size of bees can clearly have a huge effect on bee behaviour, very few aspects of how cage design affects honeybee survival and behaviour in laboratory conditions have been studied. Survival under laboratory conditions is influenced by cage design and cage size (Köhler et al. 2013). Cages constructed from a variety of different materials, shapes and sizes have been tested and cage type affected honeybee survival (Williams et al. 2013; Huang et al. 2014), but the dimensions of the cages in these experiments were not standardised and the available space per bee was not consistent. Comparing these cage designs is also problematic because the volume of the cages and the type of material (which could influence ventilation, insulation, and behaviour) were not standardised. Another part of cage design that can affect the outcomes of labratory studies is the addition of feeders (Huang et al. 2014) and wax on which bees can aggregate, although the latter is not a requirement in all types of experiments. Bees survive better when natural comb is used rather than wax sheeting (Köhler et al. 2013). However, storage of food in the provided comb could skew measurements of consumption.

This lack of standardisation in cage design complicates the comparison of results between different laboratories and could explain some of the variable results produced. As an example, in research exploring how the ratio of nutrients fed to bees affects their survival, very different dietary optima have been identified in different laboratories (Archer et al. 2014a; Archer et al. 2014b; Paoli et al. 2014). One possible explanation for these differences could be physiological differences between the subspecies of bees studied, as when honeybees of African and European origin utilise the protein in artificial

diets differently (Morais et al. 2013). These differences may also reflect differences in diet preparation, with researchers using agar-based (Archer et al. 2014a) or liquid (Paoli et al. 2014) diets. Alternatively, differences may reflect variation in the size of hoarding cages used to house bees during the experiments and the group size of the bees in the cages.

Here, we examine the effects of cage size and honeybee density on the survival and food intake of *Apis mellifera scutellata* in laboratory studies. To control for the effect of nutrition on survival, bees were provided with a choice of diets (a 50% sugar solution as well as a protein containing solution) allowing them to regulate their nutrient intake as they would in natural conditions from nectar and pollen respectively The aim is to improve our understanding of how the density of honeybees and the volume available to honeybees affect survival and consumption parameters which will also provide data to guide the design of future experiments in which hoarding cages are used.

2. Methods

2.1 Bees and cages

Bees were obtained from five colonies in the University of Pretoria apiary. Frames with sealed brood were removed from the selected colonies and taken to the laboratory where they were incubated at 35°C (Memmert GmbH+, INE550, Schwabach, Germany) and 55-65% RH. Adult bees were collected from the frames within 24 h of emergence and transferred to clear cubic, plastic cages (Polyvinyl chloride plastic gift boxes, Plastilon Packaging Company, South Africa). These were modified to house bees such that each of the four sides had 25 ventilation holes (~1 mm diameter) drilled into the sides

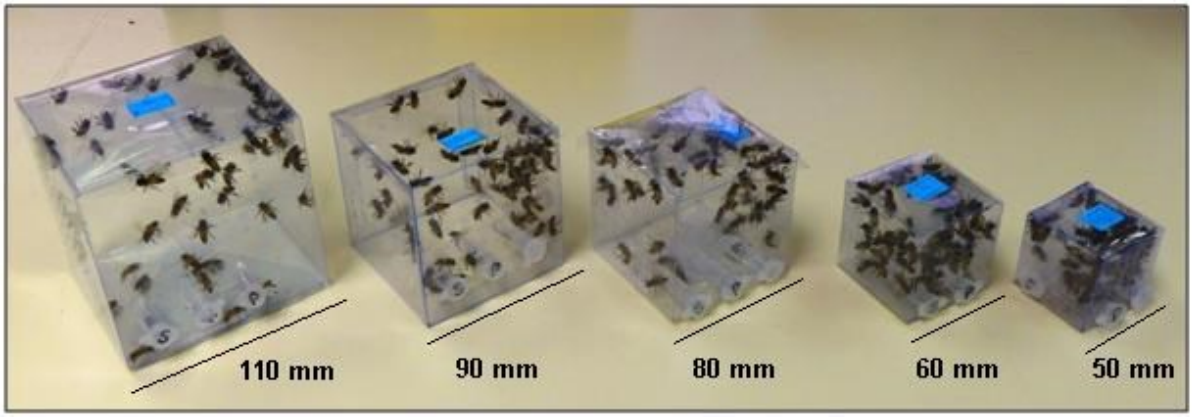

Fig. 1 Comparison of the different cage sizes with 60 bees in each cage. All cages had ventilation holes and openings for feeding tubes. Linear dimensions of the cubic cages are displayed beneath each cage.

and three larger holes (~10 mm diameter) were made on the bottom of the front panel for the two food tubes and one water tube (Fig. 1). Food was provided in Eppendorf tubes with four holes (~1 mm) drilled along the top, to allow access to the feeding solution. All cages were kept in dark incubators (Memmert GmbH+, INE550, Schwabach, Germany) and hive conditions mimicked by maintaining the temperature at 35°C as well as keeping the humidity high (55-65% RH) by placing trays of water in the incubator.

2.2 Cage size

Each cage contained bees from only one of the five colonies, and so is one replicate. Therefore, five replicates were set up for each cage volume tested (one for each colony). These cages were cubic in design and varied only in volume: 125 ml, 216 ml, 512 ml, 729 ml and 1312 ml. The space available for each individual bee in different cage sizes (ml/bee) is given in Table 1. In previous experiments we have used hoarding cages with volumes of 216 ml (Archer et al. 2014b) and 523 ml (Köhler et al. 2013) so, the cage volumes selected are representative of previously used cages as well as including both larger and smaller volumes. For each cage volume tested we included an evaporation control without any bees, giving a total of 30 cages.

2.3 Honeybee density

To determine the effect of density on honeybee survival, a hoarding cage with a set volume of 512 ml (8 x 8 x 8 cm) was selected, as this was the closest to the volume of the Perspex hoarding cages used in our previous experiments (523 ml, Altaye et al,

2010, Archer et al. 2014b). Six densities of bees were selected: 10, 30, 60, 100, 150 and 180 bees per cage. The 30 bee density was selected based on the highest survival in a previous density study (Rinderer and Baxter 1978) while 60 and 100 were selected as those are commonly used in our laboratory (Altaye et al. 2010, Köhler et al. 2013, Archer et al. 2014b). The remaining densities (10, 150 and 180) were selected to represent lower and upper extremes. For each density tested five replicates, each representing bees from a different colony was set up. Each cage contained bees from a single colony so that on colony was one replicate and five replicates were set up for each density tested. The space available for each individual bee at different densities (ml/bee) is given in Table 1. Each hoarding cage was modified from the previous design to contain five feeding tubes instead of three, and cages containing higher densities of bees (100, 150 and 180 bees) were provided with four food tubes (two containing protein solution and two sugar solution) and one tube containing water. Bees consume approximately 10-15mg carbohydrate and less than 1mg protein per day (Bosua 2017) and this experimental set up makes provision for between 25mg and 50mg dry food per bee per day. The extra feeding tubes were added to prevent crowding at the feeding tubes. Cages with the lower densities of bees (10, 30 and 60 bees) were provided with two food tubes (one containing protein solution and one sugar solution) and one water tube, as well as two empty tubes to ensure the same amount of space was being utilised by food tubes across the different cages. An evaporation control cage was set up bringing the total amount of cages to 31.

Table I The relative volume per bee and percentage survival after 14 days for each of the cage sizes and densities. Each of the different cage sizes contained 60 bees, while the different densities of bees were housed in a cage with a 512 ml volume. The total cage volume was divided by the number of bees in each cage to get the volume/bee.

Cage size (constant: 60 bees per cage)			**Density (constant: 512 ml cage volume)**		
Cage size (ml)	Volume (ml/bee)	Survival (%) ± SD	Density of (no. bees)	Volume (ml/bee)	Survival (%) ± SD
125	2.08	77.33 ± 9.66	10	51.20	62.00 ± 22.80
216	3.60	62.00 ± 10.18	30	17.07	56.67 ± 18.10
512	8.53	55.33 ± 20.61	60	8.53	62.67 ± 20.74
729	12.15	62.00 ± 12.91	100	5.12	36.20 ± 29.79
1312	21.87	60.67 ± 16.71	150	3.41	23.07 ± 41.35
			180	2.84	41.11 ± 35.47

Table II Survival was compared across different cage sizes using Gehan's Wilcoxon tests and the resulting p-values are presented here. A α value of <0.001 is taken as significant to account for multiple comparisons. Bold values are significant.

Gehan's Wilcoxon comparisons, p-values				
cage size	**216 ml**	**512 ml**	**729 ml**	**1312 ml**
125 ml	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
216 ml		**< 0.001**	0.81	0.53
512 ml			0.01	0.02

729 ml	0.50
1312 ml	

2.4 Food preparation

All cages of bees were fed 50% w/w sucrose solution as well as a protein containing solution with a P:C ratio of 1:50 (diet contained 50% water, 49% carbohydrate and 1% protein). PeptoPro^{TM} (DSM nutritional products
South Africa (Pty) Ltd., Isando, South Africa) was used as a protein source while granulated sucrose was used a source of carbohydrate. PeptoProTM is a hydrolysed form of casein and is soluble in water, making it suitable for liquid diets. The P:C 1:50 ratio was chosen as the liquid diet that led to the highest survival during a previous experiment comparing survival and consumption on a range of P:C ratios (Bosua et al.
2017).

2.5 Survival and consumption measurements

Consumption was measured across the different cage sizes and honeybee densities to control for the effects of nutrition on survival. Consumption was measured daily by weighing the food and water tubes before placing them in the cages and after removal from the cage after 24 h. To control for evaporation we placed tubes in empty cages, with one control cage for each cage size and for each bee density. For the evaporation controls the daily difference in weight between tubes (for sugar, protein and water tubes) was taken as the evaporation. The final consumption in the experimental cages was taken as the difference in weight before and after feeding to bees, minus the evaporation measured for the associated food (i.e. protein or sugar) in the same cage size or bee density. Both protein and carbohydrate consumption were calculated as mg per bee per day. The survival of honeybees in all cages was measured daily over 14 days, thus obtaining a survival

measurement that tracks the physiological transition from hive bees to foragers, by collecting and counting the dead bees in each cage. During the density experiments dead bees were replaced daily to keep the density constant. At the onset of the experiment a spare cage containing between 100 and 200 bees was set up for each of the colonies used. The bees from this spare cage were used to replace the dead bees in the experimental cages, ensuring that the replacement bees were the same age as the experimental bees. All bees still alive after 14 days were frozen at -20 °C.

2.6 Data analyses

The survival data for all experiments were analysed using Kaplan Meier survival regression, and Gehan's Wilcoxon paired t-tests were used to test for differences between densities as well as cage sizes. A Bonferroni adjusted α-value of < 0.001 was taken as significant to control for multiple testing on survival data. Cage size and colony were used as explanatory variables in the cage size experiment, while density and colony were selected as explanatory variables for the density experiment. All consumption data (protein and carbohydrate consumption) were tested for normality using the Kolmogorov-Smirnov test. Data that were non-normally distributed were analysed with main effects ANOVAs with colony and cage size or density used as explanatory variables. Differences in nutrient intake were analysed between different cage sizes and honeybee densities using Bonferroni post-hoc tests. All statistical analyses were conducted in Statistica (StatSoft, Inc., Tulsa, OK, USA; version 64).

3. Results

3.1 Cage size

3.1.1 Survival

During the course of the 14 day experiment survival of the 60 bees differed between the cage sizes (Kaplan Meier, $\chi 2 = 50.00$ df = 4, $p < 0.001$) and colonies (Kaplan Meier, $\chi 2 = 205.86$, df = 4, $p < 0.001$) (Fig. 2). The 125 ml and 512 ml cages had the highest and lowest percentage survival respectively (125 ml: 77.33 ± 9.65; 512 ml: 55.33 ± 20.60, Table I), and survival in these cages differed from that in the other cage volumes (Table II; Fig. 2). In the remaining three cage volumes the percentage survival did not differ (216 ml:

62.00 ± 10.18, 792 ml: 62.00 ± 12.09 and 1312 ml: 61.67± 16.71; Table II; Fig. 2).

3.1.2 Consumption

Honeybees were allowed to regulate their intake from two liquid diets, a protein containing diet with a P:C ratio of 1:50 and a pure sucrose diet (P:C 0:1). Colony had no significant effect on either daily (MANOVA, df = 4, F = 1.45, p = 0.1706) or cumulative (MANOVA, df = 4, F = 1.77 p = 0.0800) consumption of bees housed in different sized cages. Cage volume had a significant influence on the daily amount of protein (MANOVA, df = 4, F = 5.634 $p < 0.001$) and carbohydrate (MANOVA, df = 4, F = 5.634 $p < 0.001$) consumed by bees. Daily consumption of both nutrients was not significantly different in the 125 ml and 216 ml cages (carbohydrate: Bonferroni = 21.13, df = 4, n.s., protein: Bonferroni = 21.13, df = 4, n.s.) or between the 512 ml, 729 ml and 1312 ml

Fig. 2 Proportion surviving (± SD) over 14 days for 60 bees in cages of different volumes. Results were averaged for 5 replicate cages per volume tested, with each cage containing bees from a single colony.

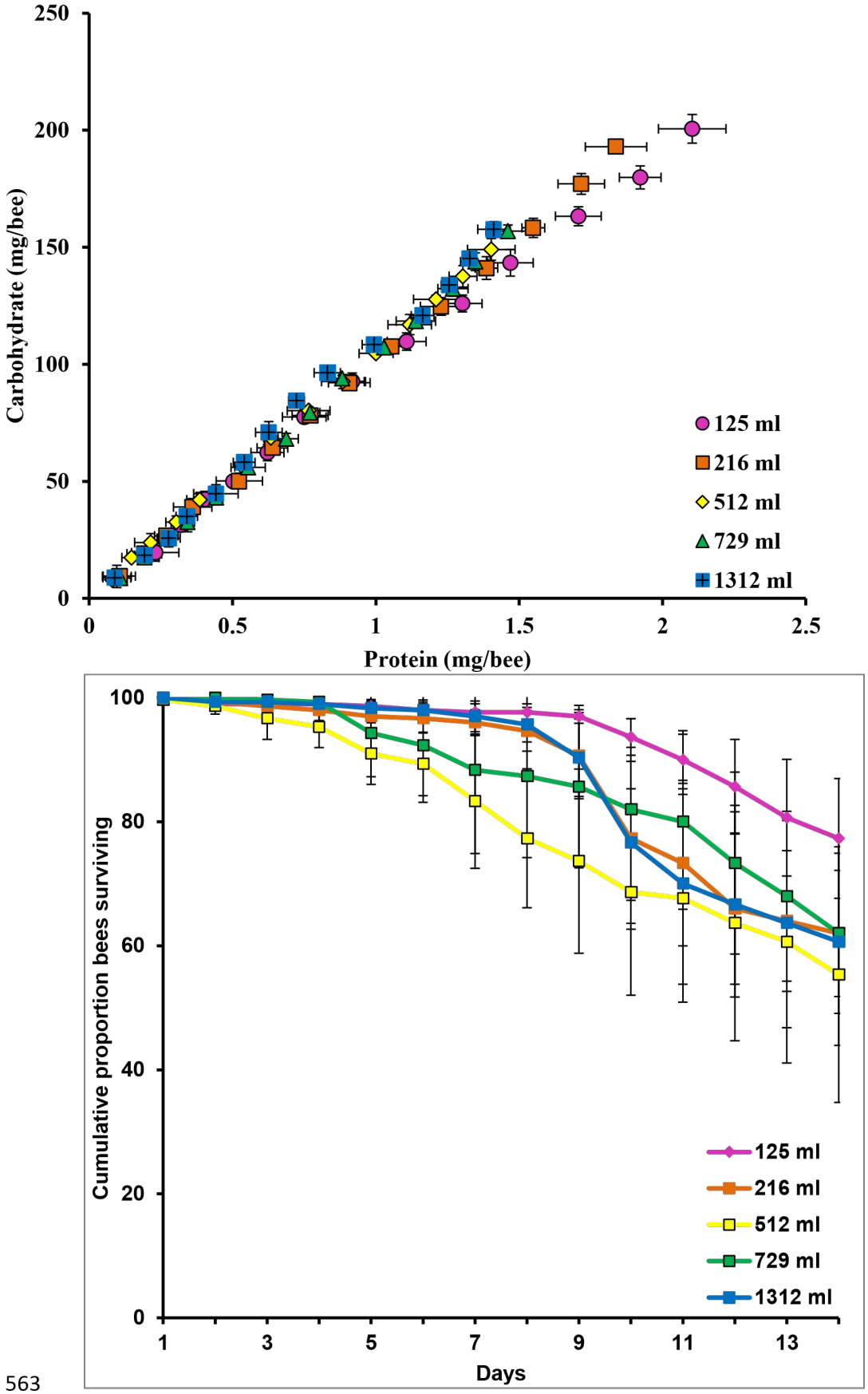

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Fig. 3 Cumulative carbohydrate and protein consumption (± SD) over 14 days for 60 bees in cages with different volumes. Results were averaged for 5 replicate cages per volume tested, with each cage containing bees from a single colony. Bees were simultaneously fed two diets with P:C ratios of 0:1 and 1:50

cages (Bonferroni = 21.13, df = 4, n.s.), however there was some significant differences between these groups.

While there was no significant difference in the 14 day cumulative carbohydrate consumption between the different cage sizes tested (Bonferroni = 3003.7, df = 4, p > 0.05) cumulative protein consumption was lower in both the 512 ml cage (Bonferroni = 0.30, df = 4, p > 0.05) and the 1312 ml cage (Bonferroni = 0.30, df = 4, p > 0.05) than in the remaining cage sizes. Nutrient intake ratios were consistent between the different cage sizes with all cages of bees converging on a similar P:C ratio of 1:105 (Fig. 3).

3.2 Density

All cages in the density experiment became dirty from traces of dried diet stuck to the sides, with the amount increasing as the group size of bees increased. In groups of 100 or more, bees produced wax which also accumulated against the sides of the cages.

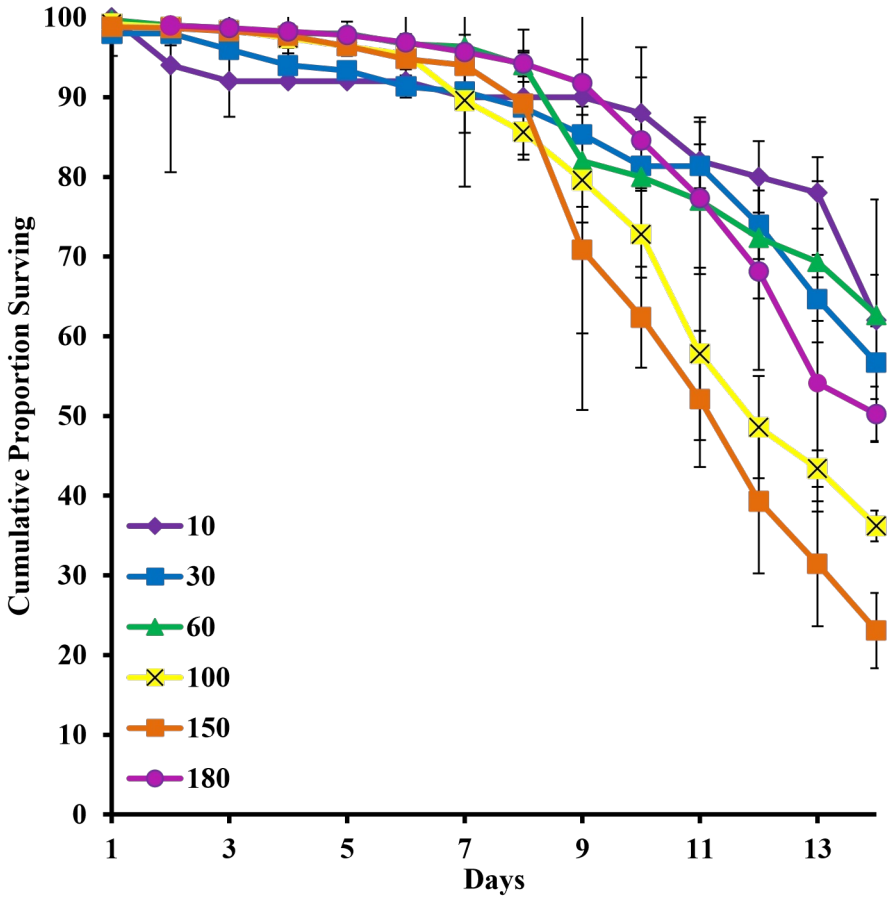
3.2.1 Survival

During the 14 day experiment honeybee survival was significantly influenced by the density of bees in the cage (Kaplan-Meier, $\chi 2 = 142.42$, df = 5, $p < 0.001$) as well as the colony (Kaplan-Meier, $\chi 2 = 544.26$, df = 4, $p < 0.001$). Bees survived longest when kept in groups of between 10 and 60 individuals (Fig. 4). Survival was not significantly different between the groups of 10, 30 and 60 bees (10: 62.00 ± 22.80; 30: 56.67 ± 18.10; 60: 62.67 ± 20.73; Gehan Wilcoxon = 0.8492, df = 5, p >0.001) while survival differed significantly between these and the remaining densities of bees (100: 36.20 ± 29.79, 150: 23.07 ± 41.35, 180: 41.11 ± 35.47: Table III; Fig. 4).

Table III Survival was compared across different densities of honeybees using Gehan's Wilcoxon tests and the p-values are presented here. A α value of <0.001 is taken as significant to account for mu ltiple comparisons. Bold values are significant.

Gehan's Wilcoxon comparison, p-values					
Density	**30**	**60**	**100**	**150**	**180**
10	0.40	0.77	< 0.001	< 0.001	< 0.001
30		0.40	< 0.001	< 0.001	< 0.001
60			< 0.001	< 0.001	< 0.001
100				< 0.001	< 0.001
150					< 0.001
180					

Fig. 4 Survival curve (± SD) over 14 days for different densities of honeybees in a standardized cage size (512 ml). Results were averaged for 5 replicate cages per volume tested, with each cage containing bees from a single colony.



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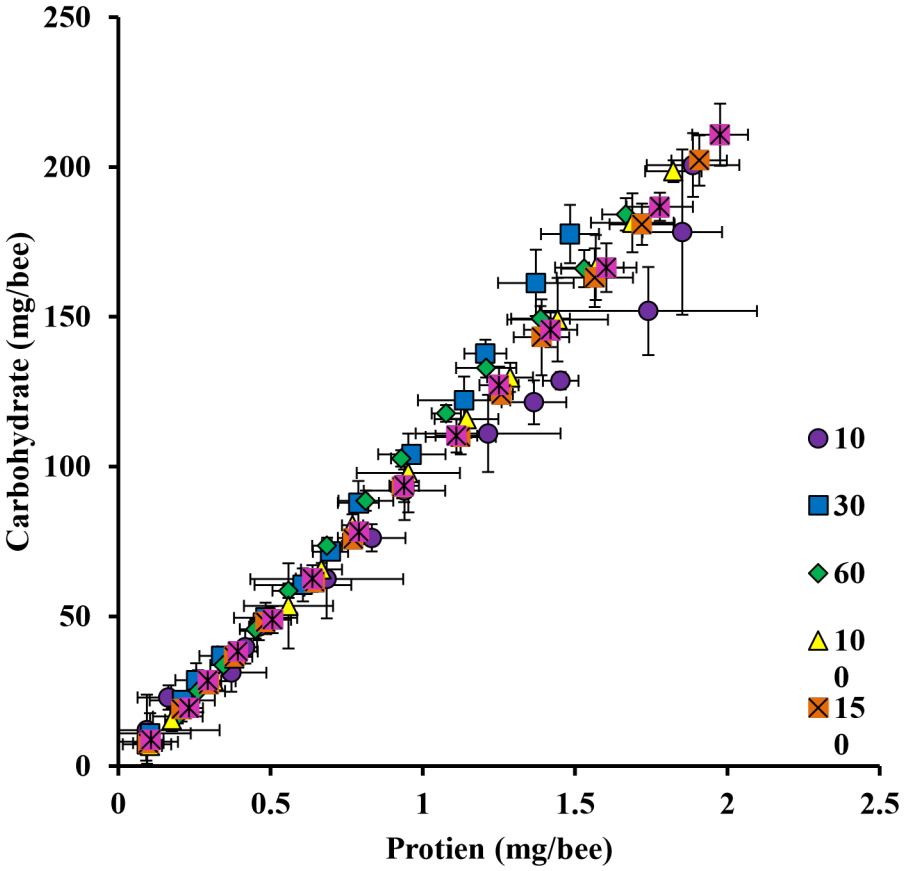

Fig. 5 Cumulative protein and carbohydrate consumption (±SD) for 5 colonies of honeybees fed a mixture of P:C 1:50 and P:C 0:1 diets for 14 days. The different colours represent the densities of bees in the cage.

To further test for the effect of colony, survival was compared between the cage size and the density experiment. Where the experimental conditions of cage size and honeybee density were similar (60 bees in a cage with 512 ml volume) survival was not significantly different (Gehan Wilcoxon = 4.3613, df = 1, p <0.001).

3.2.2 Consumption

Honeybee density affected neither daily nor cumulative consumption of either protein (daily: MANOVA, df = 5, F = 0.1587, p > 0.001; cumulative: MANOVA, df = 5, F = 0.4048, p > 0.001; Fig. 5) or carbohydrate (daily: MANOVA, df = 5, F = 65.473, p > 0.001; cumulative: MANOVA, df = 5, F = 3988.9, p > 0.001; Fig. 5) significantly. However, two of the five colonies had higher daily carbohydrate consumption than the others (MANOVA, df = 4, F= 65.473, p = 0.0172). Daily protein consumption was not significantly different between the different colonies (Bonferroni = 0.1587, df = 4, p > 0.001), but over 14 day periods three of the colonies showed higher protein consumption than the rest (Bonferroni = 0.40479, df = 4, p < 0.001).

4. Discussion

Cage size and honeybee density within a cage affect honeybee survival and nutrient intake in laboratory conditions. Cage size only affected the cumulative protein consumption in the 512 ml and 1312 ml cages where bees consumed less protein than the rest of the cage sizes. Meanwhile, honeybee density in itself did not significantly affect the amount of nutrients consumed. However, density and colony interacted in affecting protein consumption, suggesting

that there are some genetic differences in nutrient consumption in relationship to density.

However, despite these differences in consumption, bees were found to consume similar amounts and ratios of macronutrients (P:C 1:105) among the different cage sizes and different densities, thus ruling out nutrition as the main factor affecting survival in this experiment. This suggests that the differing data on nutrient regulation found in similar nutritional studies (Archer et al. 2014a; Archer et al. 2014b; Paoli et al. 2014) are not caused by differences in cage size or honeybee density, but may be due to differences in diet preparation. Indeed, the intake ratio of P:C 1:105 determined on the liquid diets used in this experiment is more similar to the P:C 1:115 determined by Paoli et al. (2014) than to the P:C 1:6.5 determined on the agar diet of Archer et al.(2014).

Bees survived best in the cage with the smallest volume, when they were in close proximity to each other and had limited space per individual. The smallest cage with 2.08 ml/bee fits within the ranges of natural conditions as described for European bees (1.9-3.8 ml/bee,Seeley and Morse 1976)) and African bees (1.89-2.1 ml.bee^{1},McNally and Schneider 1996, Vaudo et al. 2012), which supports the assumption that honeybees will perform best under conditions that mimic their natural surroundings. Survival in our experiment was very similar in cages where the available volume per bee was more than 2 ml/bee. Nest site size could also be a factor that influences site selection by swarms. A recent study on swarm occupation of three different hive types in Kenya showed that the largest hive tested (Kenyan top bar hive - 52.5 L) was the least preferred, with more swarms occupying the smaller Langstroth and log hives (McMenamin et al. 2017). Wild swarms of African bees also selected nest sites which were smaller (39 L) than the 44 L manmade Langstroth hives used by beekeepers (Vaudo et al. 2012).

Small cages have the advantage of simulating high density conditions which will affect the social interaction of thermoregulation. Younger bees (1-2 days old) have lower thoracic temperatures than older bees and when clusters form they remain on the inside of these clusters, while older bees are found on the outer edges (Harrison 1987), suggesting that thermoregulation is not as efficient in younger bees. The workers responsible for the energetically expensive thermoregulatory behaviour in the hive are usually positioned over the brood cells and receive food in the form of trophallactic secretions from donor bees (Basile et al. 2008). The more donor bees there are, the quicker thermoregulating bees can be 'refuelled' and the more efficient thermoregulation will be. The close proximity of the bees to one another could also result in social interactions such as trophallaxis occurring more frequently. Hormonal changes and subsequent behavioural development were also influenced by the frequency of worker-worker interactions in a study of bees in different group sizes (Huang and Robinson 1992), suggesting that the frequency of interactions can also be a factor in honeybee survival.

Survival was also affected by the density of bees in cage experiments, with higher survival in smaller groups (10-60 individuals), than in groups of 100-180 individuals. A different trend was found by Rinderer and Baxter (1978), where groups of 10-20 individuals had much lower survival than groups of 30-100 individuals, and survival did not differ significantly between groups once the density increased about 30 bees per cage. The most observable difference between the groups with fewer individuals and those with more individuals in our experiment was cage fouling, with the cages becoming dirtier at higher densities. The sides of the cages became caked with dried diet which the bees seem to remove without consuming, and without having a place to store it. As a result, consumption values could be overestimated in high densities of bees. Caged honeybees

may store diets when comb is provided and then consume the stored diets instead of the fresh diet in feeders (Köhler et al. 2012, Köhler et al. 2013). The amount of stored diet is also affected by the properties of the diet: less sucrose solution was stored when it had a higher nicotine concentration (Köhler et al. 2012), and our experiment would suggest that higher honeybee density is another factor that influences diet storage. Rinderer and Baxter (1978) also showed that groups of 1020 bees hoarded less diet in comb than groups of 30-50, and that groups of 100 hoarded the most diet. A trend of reduced consumption in bigger groups has been observed under natural hive conditions, where in winter consumption per bee decreased as colony size increased, without affecting the survival (Free and Racey 1968).

In the cages with the three highest densities of bees (100 bees and more per cage) the bees produced new wax which coated the sides of the cage. This is an example of a social interaction that requires certain group sizes of bees (see Table 6.1 in Hepburn et al. 2014). We have previously observed wax building in caged honeybees kept in groups of 100, which manipulated the wax sheet provided by building additional cells (Altaye et al. 2010). The presence of wax can influence bee behaviour in the hive and can trigger temporal polyethism. Wax deprivation induces bees to abandon nurse bee duties and become foragers or wax producers (Fergusson and Winston 1988). Wax has an important role in the hive; in addition to being used as a substrate to store nutrients and house brood, it also absorbs cues and food scents in the same manner as the hydrocarbon based cuticle of workers, which then aids in nest mate recognition of returning foragers (Breed et al. 1988). Experimental designs normally include adding a piece of wax to the cage, in the form of either wax sheeting or wax comb removed from the hives (Altaye et al. 2010, Köhler et al. 2013 Archer et al. 2014). The addition of wax in the cage seems to increase

survival, but bees will also use this space to store some of the diet they were given, and if consumption is measured this may skew the results
(Köhler et al. 2013).

The seeming contradiction that the highest survival was found on the lower densities of bees as well as the smallest cages suggests that it is not only the volume per bee that plays a role, but also the social interactions and the cumulative effect of diet hoarding. Two factors differed between high density cages and large cages with a low volume per bee, namely cage fouling as a result of hoarding behaviour and wax production. Cage fouling due to hoarding behaviour was observed in the high densities of bees, but not in the small cages, even though in both situations the available volume per bee was low (<3 ml.bee). This does not necessarily mean that the bees hoard more at higher densities, but that there are more bees hoarding at the same rate. Rinderer and Baxter (1978) observed that the comb in cages of 100 bees contained more hoarded diet than the other group sizes, even though they hoarded diet at similar rates. Therefore, even though bees are in close proximity to each other in both the highest densities and the smallest cage volumes, more diet and more feeders were available to more bees in the high density experiment than in the cage size experiments and this could have caused cage fouling to become a significant factor in reducing survival. Since wax production requires groups of 100 or more bees (Hepburn et a.l 2014), this was observed only in the high densities and not in the low volume experiment, although once again the bees were in close proximity to each other in both situations. The energetic cost of producing wax (Hepburn et al. 2014) could contribute to the reduced survival in the high densities and explain why the bees in the small cages did not suffer the same adverse effects.

We have shown that cage volumes that allow bees the same amount of space as naturally selected nest sites yielded the best survival. We have also shown that while bees prefer to be in close proximity to each other, large group sizes in the laboratory will lead to different social interactions that should be taken into consideration. Honeybees will try and adapt their environment around their requirements by thermoregulation or wax building, and will suffer increased mortality when they are unable to do so.

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Author Contributions

HJB, SWN and CWWP conceived this research and designed experiments; CRA and CWWP participated in the design and interpretation of the data; HJB performed experiments and analysis; all authors wrote and revised the paper. All authors read and approved the final manuscript.

Conflict of interest

"The authors declare that they have no potential conflict of interest in relation to the study in this paper"

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