Foraging decisions in different landscapes and the use of associative memory for the locust *Locusta migratoria*

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Abstract

The foraging behaviour of herbivorous insects is influenced by many different factors, of which a few have been studied in this project. These include the nutritional value of the available food resources, the frequency at which they occur and their spatial distribution within the environment. Foraging behaviour has a direct impact upon the strategies that individual plants use to escape herbivory, such as spines and poisonous compounds. Foraging will also affect the distribution of plants in time and space, which may in turn offer opportunities for plants to avoid herbivory. This project studies the effects on the foraging behaviour of a herbivores locust species *Locusta migratoria*, when faced with low frequencies and different distributions of a complementary food within an environment dominated by low-quality food resources The limiting food was a high-protein artificial diet. A high carbohydrate diet was used as low quality food. The arena contained 49 dishes, the minority of these dishes contained P-diet, the rest contained C-diet. Three different arrangements were tested: 5 P-dishes clumped together, 4 P-dishes clumped together and 1 isolated and 7 P-dishes over-dispersed throughout the arena. The first and last arrangements were designed to test the effect on both foraging behaviour and the food resources of having limited resources clumped, while the second asked whether it was advantageous for limiting foods to be separated in an environment in which others were clumped. No differences in nutrient intake and foraging behaviour were found between the three treatments. The amount eaten from the isolated dish in treatment 2 was also not shown to be different from the other P-dishes. The locusts in the third treatment however seemed to eat more protein than in the other treatments, but this was not proven to be significant due to small sample sizes. The average loss per P-dish and the portion of P-dishes eaten severely from was also the same for all the treatments. These results suggest that it does not matter which of the strategies are used by plants providing a limiting resource. In addition the spatial associative memory of locusts was studied. Locusts were left to forage for 2 days in an arena with 5 clumped P-dishes and 44 C-dishes. The walls had different visual cues that the locusts could use to associate with the location of the P-dishes. On the 3rd day the locusts were taken out of the arena and left to feed on C-diet only for 4 h. The locusts were reintroduced in the arena after this period and their movement patterns recorded.

The results suggest that some of the locusts remembered the site of the protein dishes, but sample sizes were too small to reach significance.

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1. Introduction

1.1 Feeding behaviour in locusts

When studying the foraging behaviour of an animal a variety of factors must be taken into account. These include the various nutrients the animal has to obtain to maintain good health and to maximise evolutionary fitness, the nutritional state of the animal at the time, the optimal nutritional state the animal can obtain, which foods should and should not be eaten, and the consequences of eating various foods. A model that integrates these factors to describe the nutrient exchanges between organisms and their environment is the geometrical framework (GF), developed by Raubenheimer & Simpson (see Raubenheimer & Simpson 2002, Simpson et al., 2004). An advantage of this model is that it can deal with two or more food components simultaneously.

In this project the two food components protein and carbohydrate will be used to study the foraging behaviour of the locust. Protein and carbohydrate are the two most important macronutrients for which herbivores regulates intake by varying food choice (Raubenheimer & Simpson, 2002). The optimal nutrient (in this case protein and carbohydrate) intake requirements can be combined as a point in a nutrient space (fig. 1). This 'intake target' represents the animal's needs for both nutrients over a given period. A variety of studies show that locusts actively regulate to this intake target; instead of maximizing intake they balance ingesting excesses and deficits of the various nutrients (Simpson *et al.*, 2002; Raubenheimer & Simpson, 2003; Behmer *et al*., 2003; Behmer *et al*., 2001; Zee *et al*., 2002).

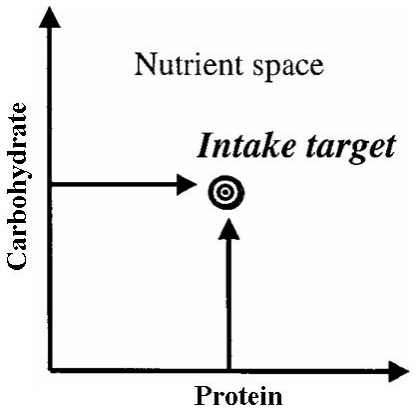


Figure 1. Combining the optimal intake points for protein and carbohydrates gives the intake target in nutrient space (Raubenheimer & Simpson, 2002).

When animals encounter different foods they will decide what and how much to eat from each food source based on two major aspects, the amount of the various nutrients and the proportions of the nutrients in the food. In addition, the presence of toxins or allochemicals (Raubenheimer, 1992; Simpson & Raubenheimer 2001; Behmer *et al*., 2002), amount of indigestible cellulose and the accessibility of the food source are also important, but will not be discussed in this project. A food source that contains a perfect combination of nutrients for the animal to reach its target intake is called a 'balanced food' (fig. 2a). Unbalanced food on the other hand does not contain the perfect combination of nutrients, and an animal eating from this will never reach the target intake but ends up with excesses and deficits for the various nutrients (fig. 2b). However, different unbalanced foods can be combined to reach the target intake, in which case the food sources are called 'complementary foods' (fig. 2c) (Raubenheimer & Simpson, 2002, 2004a,b).

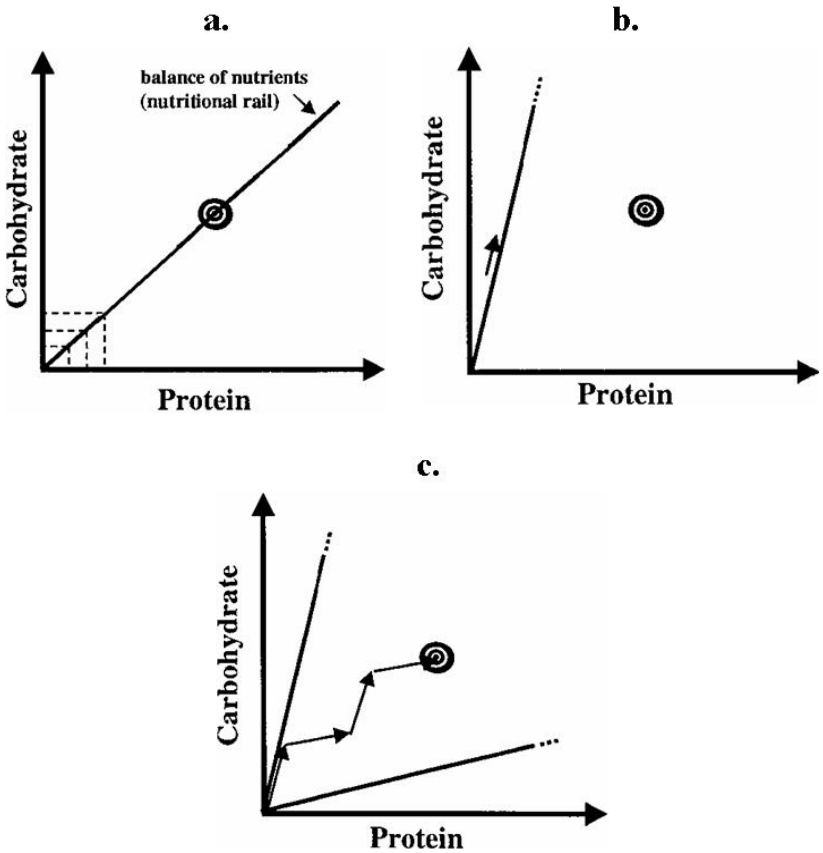


Figure 2. The nutritional rails represent the balance of the nutrients in different foods. (a) Shows a balanced food, while (b) shows the rail of an unbalanced food. The animal feeding on this food source will never reach its target intake point. When two unbalanced food sources are combined, the animal can reach the target intake by eating from both foods (c). These food sources are now called complementary foods (Raubenheimer & Simpson, 2002).

The target intake and nutritional regulatory capacities of a locust can be obtained by presenting different foods to the insect and measuring the intake from each dish. The target intake can be obtained by measuring the intake of a locust from two or more complementary foods. Testing whether the locust actively regulates this target intake and its nutritional state can be done by presenting different complementary foods to different individuals. If regulated intake is involved, all animals would arrive at the same point of nutrient intake. If the animals on the other hand eat randomly then they will end up at different nutrient intake points (Raubenheimer & Simpson, 2002, 2004; Simpson et al., 2004).

Animals also have to regulate their nutrient intake when faced with nutritionally unbalanced food. They do this according to the 'rule of compromise', which means that they have to decide how much excess of one nutrient and deficit of the other they will tolerate (fig 3a). Which rule of compromise a locust uses can be tested by first obtaining the target intake, and then exposing insects to a different unbalanced food source. This would generate a nutrient space with different nutrient rails that represent the unbalanced food sources (fig 3b). On each rail an intake point will be present, which is the point that benefits the animal most when eating that unbalanced food. Together these points form an intake array, which represents the rule of compromise the animal uses when faced with different unbalanced foods (Raubenheimer & Simpson, 2002, 2004).

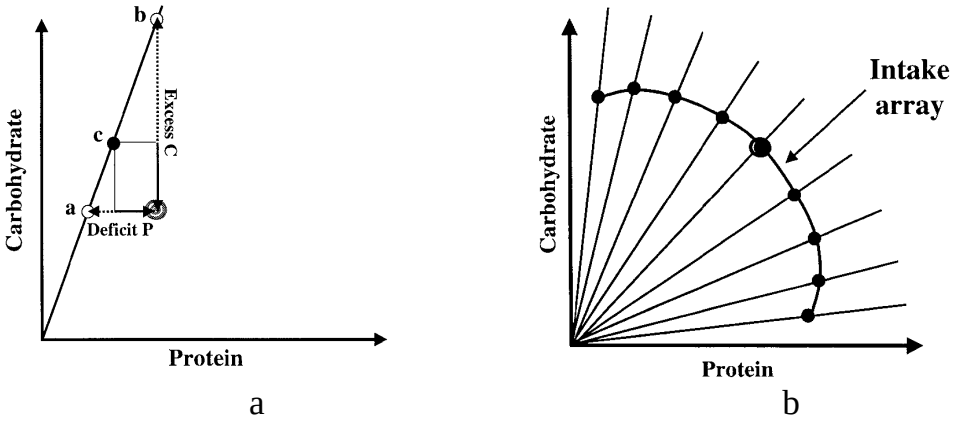


Figure 3. When faced with a nutritional unbalanced food, animals are forced into a trade-off between eating too much of some nutrients, and eating to little of others. At point a the animal will have the right carbohydrate intake, but a deficit in protein intake. At point b the animal has eaten too much carbohydrates, but has the right protein intake. Point c is the trade-off point, where the animal has both a deficit for protein and an excess for carbohydrates. (b) The intake array is obtained by combining the trade-off points for different unbalanced foods. The shape of this array then gives information about the rule of compromise used by the animals (Raubenheimer & Simpson, 2002).

1.2 Locusts foraging behaviour differences between phase and species

The strategies that locusts use to defend their optimal nutritional state vary between different species and between phases. Nutrient intake strategies have been studied for the species *Schistocerca gregaria* (Zee *et al*., 2002; Raubenheimer & Simpson, 2003; Simpson *et al*., 2002) and *Locusta migratoria* (Behmer *et al*., 2001; Behmer *et al*., 2003; Raubenheimer & Simpson, 2003). *S. gregaria* is also referred to as the African desert locust, and is a generalist feeder. Like *L. migratoria*, it can phase change from solitarious to gregarious and vice versa. The foraging strategies of these species have been tested when faced with different frequencies of balanced, unbalanced and complementary foods (Behmer *et al*., 2001; Raubenheimer & Simpson, 2003 Simpson *et al*., 2002), single unbalanced foods (Raubenheimer & Simpson, 2003; Simpson *et al*., 2002), and various distances between two complementary foods (Zee *et al*., 2002; Behmer *et al*., 2003). The foods in all these tests were a combination of protein and carbohydrate, which are the main macronutrients for which the animal regulates its intake.

The target intake of the locusts was shown to vary between species (Raubenheimer & Simpson, 2003), but not between phases (Simpson *at al*., 2002). *S. gregeria* ingested more protein than did *L. migratoria*, although the nutrient composition of the animals remained indistinguishable. This suggests that *Locusta* has a higher retention efficiency compared to *Schistocerca* (Raubenheimer & Simpson, 2003). The fact that the target intake did not differ between the two phases of *Schistocerca* is unexpected, but might be caused by the experiment itself, where the animals were observed alone in the same size arena. This restricted the gregarious locusts in their mobility and might also have partially induced behavioural solitarisation (Simpson *at al*., 2002).

Schistocerca and *Locusta*, as well as the gregarious and the solitarious phase animals of *Schistocerca*, showed active regulation of nutrient intake when faced with nutritionally complementary foods (Behmer *et al*., 2001; Raubenheimer & Simpson, 2003 Simpson *et al*., 2002). Behmer *et al.* (2001) demonstrated that *Locusta* balanced its intake regardless of the frequency of foods in the environment, demonstrating negative frequency-dependent selection (i.e. eating more from rare than from common complementary foods to maintain their intake target). However, when the locusts were faced with different frequencies of both balanced and unbalanced food, they

never reached a balanced intake because they ate a small proportion of total intake from the unbalanced foods. Whether this was part of an adaptive strategy in which the environment is continually sampled, or reflected sensory 'mistakes', is not clear.

Switching behaviour between food sources was also observed by Zee *et al*. (2002). Gregarised *Schistocerca* nymphs were shown to switch more between complementary foods that were close together (25 cm) compared to solitarious nymphs. However, switching was not solely dependent on balancing nutrient intake since the number of switches made was the same for nymphs that could choose between two of the same food sources.

When faced with a single unbalanced food *Schistocerca* and *Locusta* showed different intake strategies. *Schistocerca* ingested a more extreme excess of protein, and overall ingested more protein and carbohydrate than *Locusta*. This difference diminished when the proportion of carbohydrate in the food increased. *Schistocerca* is able to allocate higher levels of protein when fed with protein deficient foods. *Locusta* however, showed a higher efficiency for the conversion of nitrogen. Overall, it was shown that the intake array of *Locusta* was more arc-shaped, whereas the intake array of *Schistocerca* was more linear (Raubenheimer & Simpson, 2003). These nutrient regulation patterns can be well correlated with the fact that *Schistocerca* is a generalist feeder whereas *Locusta* is a specialist feeder. A generalist feeds on a variety of plants in order to obtain the optimal nutrient intake. It will therefore be more willing to overeat a nutrient because the probability of encountering a complementary food source is relatively high, compared to a specialist feeder. A specialist only feeds on one type of plants, and is therefore dependent on the nutrient composition of that plant. In order to deal with changes in the nutrient composition of the plant it adopted a more efficient nutrient retention (Raubenheimer & Simpson 1999). These locusts regulate their protein intake more than carbohydrate, since proteins can be deaminated to substitute carbohydrate, whereas the reverse is not the case (Simpson & Abisgold, 1985; Raubenheimer, 1992; Zee *et al*., 2002).

The same differences in nutrient balancing strategies were observed between the gregarious and the solitarious phases of *Schistocerca* (Simpson *et al*., 2002). The gregarised nymphs showed a linear intake array when faced with different unbalanced foods, which resembled the intake strategy of a generalist feeder. The solitarious phase on the other hand, showed a more arc-shape intake array, resembling the specialist feeder. The solitarious nymphs also developed more quickly on all diets

(except for the extremes where development time was the same in both phases), indicating a higher efficiency of converting of the ingested nutrients to growth. The gregarised nymphs showed a higher intake of protein, however, both phases ingested the same amount of protein, suggesting that the gregarious nymphs utilised protein less efficiently, compared to solitarious nymphs.

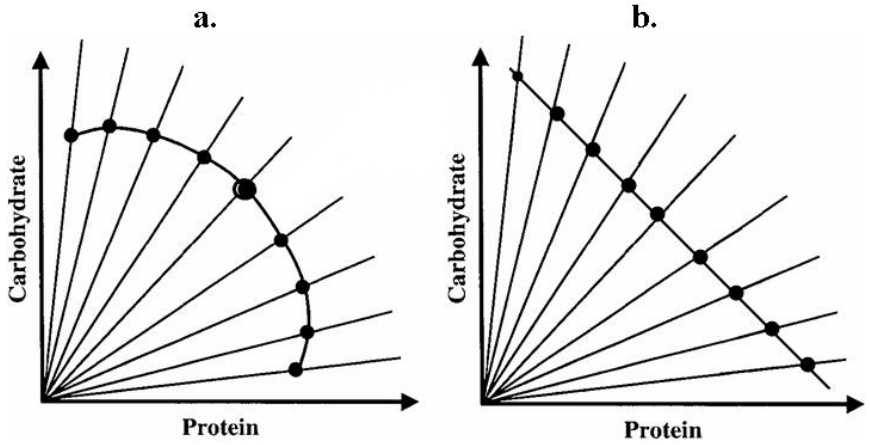


Figure 4. The intake arrays of the specialist feeding locusts *Locusta migratoria* (a) and the generalist feeding locusts *Schistocerca gregaria* (b). The same arrays apply for the solitarious (a) and gregarious (b) phase nymphs of *S. gregaria*, respectively (Raubenheimer & Simpson, 2002).

1.3 Associative learning in the African migratory locust

The first research that showed associative memory in locusts was done by Goldsmith *et al*. (1978) where they demonstrated that locust nymphs and adults can learn to associate leg extension with an electrical shock, and avoid the shock by maintaining leg flexion. This associative memory lasts for about 3 days in both the 5^{th} instar locusts and the adults, and was show to survive metamorphosis into the adult stage.

Now, several studies have show that locusts use associative memory for the regulation of nutrient intake. They are able to associate olfactory (Simpson &White, 1990) and visual stimuli (Raubenheimer & Tucker, 1997) with the quality of foods and the location of water (Raubenheimer & Blackshaw, 1994). Learned associations between unspecified cues and the nature of phytosterols in leaves (Champagne & Bernays, 1991; Behmer *et al*., 1999) and between visual cues and food availability (Bernays & Wrubel, 1985; Holliday & Holliday, 1995) have also been demonstrated.

Locusta migratoria has been used in several of these studies, which show that it can learn to associate food quality with different environmental cues. Simpson & White (1990) trained *Locusta* nymphs to associate protein and carbohydrate with

different odours. These locusts were then deprived of either protein or carbohydrate for 4 h, and put in an area that contained both odours, but no food. The locusts that were deprived of protein showed a high preference for the odour that they were learned to associate with the presence of protein food. Locusts deprived of carbohydrate showed no preference for either of the odours. The odours used in this research were originally repellent to the locusts, however, association with protein presents neutralised this effect. It was shown that locusts deprived of protein were never attracted to the protein associated odour, but were merely repelled by the odour associated with the carbohydrate. Because locusts regulate their protein intake more than their carbohydrate intake the effect of the odour associated with protein was neutralised, which did not happen for the carbohydrate associated odour.

Raubenheimer & Tucker (1997) used visual stimuli (green and yellow), which are relatively neutral. Nevertheless, naïve locusts showed a slightly higher preference for the yellow colour, but this was of no significant influence to the results. Locusts were trained in the same manner as was done by Simpson & White, but instead of odours, colours were associated with the two foods. Locusts showed associative memory for both colours and nutrients, moving to the colour that had been associated with the nutrient for which they had been deprived. A slight difference was found between males and females. Females showed a slightly stronger memory when they were deprived of protein, whereas males had a stronger memory for carbohydrate.

1.4 Herbivore foraging and plant distribution

When foraging in a natural landscape, herbivorous animals face a highly diverse distribution of plants. Within this landscape the animals have to make a range of decisions, including from which plants they should and should not eat, the size of their foraging area and when to leave to forage in another area. These questions have been studied mainly among mammalian herbivores, mostly by looking at departure decisions when foraging over different spatial scales. These scales are defined as the bite, feeding station and patch (review: Searle *et al*., 2005). The bite is characterised as the part removed from a plant within a single bite (Kotliar and Wiens, 1990). A collection of bites, with all bites being removed without the movement of the animal's forelegs is called the feeding station, and a collection of feeding stations is referred to

as a patch. A patch again is separated from its surroundings by changes in intake rates that are gained as the animals travel between the patches (Senft *et al*., 1987; Bailey *et al*., 1996).

Herbivores select for the most 'palatable' plants, and plants try to avoid being damaged by herbivory. This interaction can influence the composition of plant communities and the behaviour of the herbivorous animals (Augustine & Mc Naughton, 1998). Herbivores make foraging decisions based on the collection of plants in patches, and can be selective within or between patches (Bergvall *et al*., 2006). Selection between patches mostly occurs when the different plants in a patch can be visually distinguished, but an animal can also decide to leave a patch after foraging when the density of unpalatable plants is too high. These animals are not selective within patches, so they will eat equal amounts of all the plants in one patch. Plants can adjust to this by gaining protection from the defence traits used by neighbouring plants. Palatable plants in a patch with many unpalatable plants will therefore escape being eaten, which is called associational defence (reviewed by Milchunas & Noy-Meir 2002). Unpalatable plants in a palatable patch, however, will be damaged more, which is called associational susceptibility (Hjältén *et al*., 1993). Both associational defence and susceptibility can be caused by herbivorous insects that are selective between, but not within patches (Hambäck *et al*., 2000; White and Whitham, 2000). In general, unpalatable plants should gather in patches to escape being eaten, while palatable plants should not.

When herbivores on the other hand select within patches, plants have to use the opposite strategy to avoid being eaten. Less palatable plants should now grow in a patch with a more attractive plant which is preferred by the herbivores. The less attractive plants will escape being attacked, since the herbivores prefer to eat from the attractive plant. This strategy is called the attractant decoy hypothesis, and has been suggested to apply to insect herbivory (Atsatt and O'Down, 1976). It has also been used to save crops in a field by planting another crop that acts as an attractant decoy and will lead the insects away from the crops in the field (Hokkanen, 1991).

What is also important in the decision of herbivores to leave the patch where they are feeding is the distance to other patches. Different studies have demonstrated that herbivores remain longer in profitable patches, and that their residence time increases as the distance to other patches increases (Visås & Sæther, 1987; Distel *et al*., 1995; review: Searle *et al*., 2005). It has also been shown that the migratory locust

L. migratoria tend to remain longer at a feeding site when the distance to other feeding sites increases. It was predicted that when the distance between the feeding sites would continue, it would reach a point where it would no longer be cost-effective for the locucts to travelto the other feeding site, thus forcing them in decisions of habitat choice (Behmer *et al*., 2003). On the other hand, when the distances between the different feeding sites are small and costs en benefits for travelling between these food sources are low, it becomes hard for herbivores to decide which behaviour would be most beneficial (Roguet *et al*., 1998).

2. Research aims

Nutrient intake and foraging behaviour in locusts have been studied extensively, however only in small arena's with relatively few environmental factors to which the locusts could respond. The aim of this project was therefore to look at the effects on foraging behaviour and nutrient intake by locusts, and in turn their impacts on the intensity of foraging from individual food items, in a large arena with different food sources arranged in 3 different patterns. Since locusts are more selective for their protein intake than for their carbohydrate intake, high protein diet was used to represent the limiting, complementary food. The P diet was present in low frequencies, whereas the high carbohydrate diet was abundantly present in the arena. The P-diet dishes were present in 3 different arrangements; 5 dishes clumped together, 4 dishes clumped together and one isolated, and 7 dishes over dispersed throughout the arena. The behaviour of the locusts was monitored, and it was studied which dishes they choose to eat from, and if they selected a certain part of the arena as a foraging area. This information could provide ideas on what arrangement would be most beneficial for the plants, and how this would help them to escape being eaten by the locusts.

Second, it was studied whether locusts are able to associate the location of the P-diet with visual cues present on the walls of the arena. It has been shown in other studies that they do develop associative memory, however, these studies only use relative simple arenas, where the locusts were faced with two options only. In their natural environment locusts will be faced with much more complex stimulus sets. For this experiment the locusts in the '5 clumped' arrangement were used, after they had foraged within that environment for 2 days.

In general the project was designed to answer the following questions:

1. How do locusts adjust their foraging behaviour in a large complex arena with different food sources and different arrangements of these foods?
2. What is the implication of such foraging patterns on levels of herbivory experienced by food plants?
3. Can locusts associate the visual cues in their environment with the location of high quality (in this case, complementary) food sources?

3. Materials and methods

Locusts

Locusta migratoria 5th instar nymphs were used in this project. The insects were supplied by the Taronga Zoo in Sydney where they were crowd-reared for many generations. They were kept under crowded conditions and were fed on seedling wheat. In all experiments gregarised *Locusta migratoria* nymphs with the same age and nutritional state were used. To achieve this, freshly moulted 5th instar locusts were put in a 11 cm x 17 cm box with two artificial diets (P and C) from the day that they moulted (day 0) till the next morning when they were introduced into the experimental arena (day 1). The locusts were weighed on day 0, day 1 before the start of the experiment, and at the end of the experiment (day 3). Only locusts that fell between a critical weight range on day 0 were included in the experiment: 0,445 – 0,609 g for females, and 0,327 – 0,470 g for males. All experiments were carried out in a controlled temperature room at 29-31 ^{o}C with a 15:9 h light:dark period.

Diets

During the experiment the locusts had access to both high protein diet (P) and a high carbohydrate diet (C). The high protein contained 35% protein and 7% carbohydrate (p35:c7), the high carbohydrate food contained 7% protein and 35% carbohydrate (p7:c35). The diets were prepared according to the protocol in Appendix A (p. I). All dishes containing diet were weighed before the start and at the end of the experiment, and dried for 24h in a 30^{o}C oven before weighing.

Arena

The locusts were observer in an 80 cm x 80 cm Perspex arena. The walls of the arena were 30 cm high and coated with Fluon (Teflon suspension) to prevent the locusts from climbing. The arena contained 49 food dishes (diameter: 2 cm) and 36 water sources (diameter: 1 cm) which were placed in holes in the floor of the arena. For the food dishes plastic lids were used, and the waterholes contained dental sticks immerse in water containers. The food dishes and water holes were equally distributed in the arena, each food dish being 10 cm apart from the next food dish, and each water hole being 10 cm apart from the next water hole (see fig. 5a). Food and water could be

replaced from below the floor of the arena without disturbing the insect. The animals could rest on metal perches placed in the corners, against the walls, and in the middle of the arena (see fig 5a). Different visual cues were present on the walls of the arena that could potentially be used by the locusts as orientation markers. Figure 5b shows the different shapes used, all shapes had the same green colour. Shape 1 was always present on the north wall, shape 2 on the west wall and shape 3 on the east wall. The wall between 7.1 and 7.7 remained unmarked. The position and the behaviour of the locust was recorded and analysed using Ethovision software (Noldus Information Technology b.v, Wageningen, The Netherlands). The arena was divided into 49 zones, each zone having a food dish in its centre. The zones were named by their xy coordinates. The locations of the perches were made hidden zones.

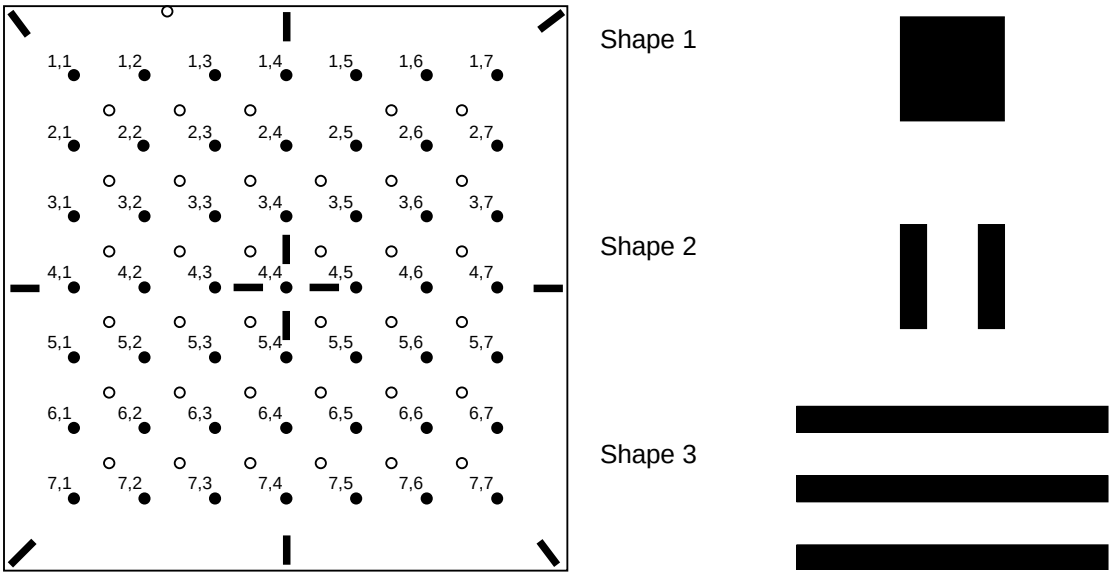


Figure 5. The set up of the arena (a). Black dots represent food dishes, white dots the waterholes and the black stripes are the location of the perches. Three different visual cues were used (b). Shape 1 was always on the north wall, shape 2 on the west wall, and shape 3 on the east wall. The south wall remained blank.

The experiments

Three different treatments were designed to investigate the influence of spatial distribution of food sources on the foraging behaviour of locusts. In all treatments the P diet was used as the limiting complementary food resource, being scarce in an environment of many carbohydrate food sources. The order in which the trails of the 3 treatments were provided, as well as the arena in which they were carried out, were randomized.

Target intake

The target intake of freshly moulted 5^{th} instar *Locusta migratoria* was tested. Five locust were used in this experiment. All locusts were weighed on day 0, 1 and 3, and had access to P and C diet for two days. Diets were weighed before and after the experiment to estimate intake. Locusts were kept in small boxes (11 cm x 17 cm = 187 cm^2).

Treatment 1

In the first treatment, 5 P dishes were clumped together, centred on a random spot in the arena. The other 44 dishes contained C diet. Six positions for the clumped P dishes were analysed, where the middle dish was randomly allocated and the other four were placed around this spot (see Appendix B, p. IV for the locations of the P dishes in each trail). The central dish position and the dishes immediately next to the wall were excluded as possible positions for the middle dish. One locust was used per experiment, and was weighed before introducing it into the arena. Each individual animal was left to feed in the arena for 2 days, whilst filming its behaviour. On day 3 the locusts were removed from the arena and weighed. They were left for 4 h in a box where they could feed on the C diet only. The dishes in the arena were put in the oven for drying and weighing, and replaced by dishes containing C diet only. After 4 h the locusts were weighed again and introduced in the arena, where they were left to feed until the next morning, whilst filming their behaviour. On the fourth day the locusts were taken out of the arena and weighed a final time. The dishes were dried for 24h and weighed.

Treatment 2

The second treatment exposed the insects to an environment with 5 P dishes of which 4 were clumped together, and one was in a location isolated from the other 4. The position of the middle P dish in the clump of four, as well as the arrangement of the dishes in the clump, was randomised, excluding the central dish and the dishes next to the wall (see Appendix B, p. V). The 5th dish was positioned as far away from the clump as possible, without making use of the rows and columns 0 and 7. Six different arrangements were tested with one locust each. The animals were weighed before introducing them into the arena, and left to feed for 2 days. The 3rd day they were weighed again. The dishes were put in the oven for 24h and weighed after.

Treatment 3

Finally the influence of over dispersed limiting complemtary food (represented by the P diet) on the foraging behaviour was tested. Seven P dishes were arranged in the arena in such a way that the distance between them was kept at maximum. The position of the first dish was randomised, the other seven were then placed so that the individual distances were maximal (see Appendix B, p. VI). Six different arrangements were made, and the central dish and the dishes next to the wall were included as possible positions for the first dish. Again one locust was used per trail, and all locusts were weighed before and after the experiment, as well as the food dishes. The animals were observed for 2 days.

Recording foraging behaviour

There was access to two arenas during the project I had, making it possible to run two trails at the same time. The foraging behaviour of each individual in the arenas was recorded using time-lapse cameras. Arena 1 was filmed by a Sony?, arena 2 bij a Canon?. The interval time used was 30 s, recording time 1 s. The recording mode was LP, making the length of each tape 90 minutes. Ethovision software (Noldus Information Technology b.v, Wageningen, The Netherlands) was used for the analysis of the experiments. The tapes were played back in Ethovision using a Sony HDR-HC3E HDV camera.

4. Results

4.1 Nutrient intake and food consumption

Nutrient intake and food consumption were measured to see whether the locusts were able to regulate their nutrient intake in all three treatments. The intake points chosen by the locusts in each of the treatments are shown in figure 6. The control experiment was done to obtain the basal nutrient intake of the locusts. Nevertheless, these measurements differed so much from the data obtained by the treatments, that it was decided to leave it out. The intake point of the 3 treatments seem to differ in amount carbohydrate and protein eaten, but this was not proven to be significant (MANOVA $F_{2,\ 15}$=1.40, P=0.28). The protein intake only is also not different between these experiments (ANOVA, $F_{2,\ 15}$=3.13, P=0.073). Intake points could also be misjudged since it was not possible to correct for errors accumulated through measuring 49 food dishes. The average weight gain of the locusts is shown in figure 7. In treatments 1 and 2 the average weight gain is similar, however in the 3rd treatment the weight gain of the locusts seems higher. But again statistics showed that this difference was not significant (ANOVA, $F_{2,\ 15}$=3.17, P=0.07).

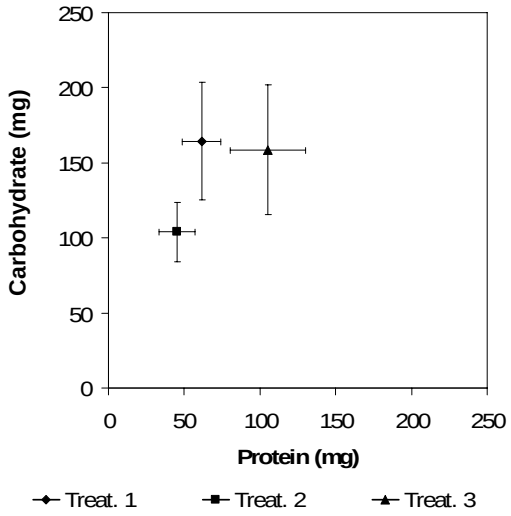


Figure 6. The mean ± SE protein and carbohydrate intake of the locusts in treatment 1 2 and 3 and the control group.

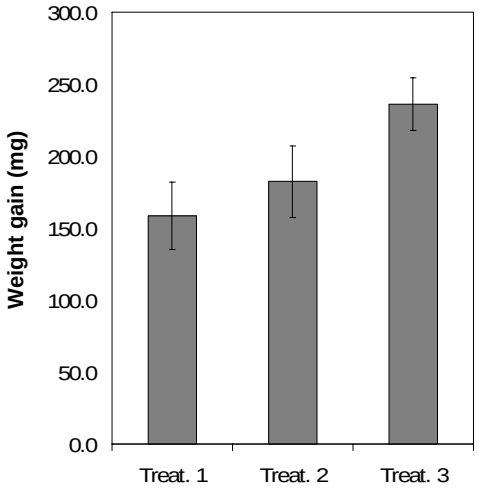


Figure 7. The mean ± SE weight gain after two days of the locusts in treatments 1-3 and the control group.

Because the amount of spillage could not be determined accurately it was not possible to say precisely how much was eaten from each dish. It was therefore decided to examine the average weight loss per dish per diet, and the number of dishes from which the loss was higher than the average loss per dish per diet. That means that for each trail the mean loss per C dish and per P dish was calculated. These number were then used to determine the portion of the dishes where the loss > mean loss, for each diet separately. The average weight loss per dish is always higher for the P diet compared to the C diet, as is shown in figure 8. There is no significant difference between the C diets for the 3 treatments, but the average loss per P dish seems to be higher in treatment 3. This was however, not significant (ANOVA, $F_{2,\ 15}$=2.15, P=0.15). The mean $\pm$ SE loss for the isolated P-dishes in treatment 2 was found to be 10.5 $\pm$ 7.87 mg, which is not different from the other P-dishes. Figure 9 illustrates the portion of food dishes where the loss > mean loss per dish for each treatment. There is no significant difference between the treatments for the portion of C dishes, and the portion of P dishes is is also the same in all treatments.

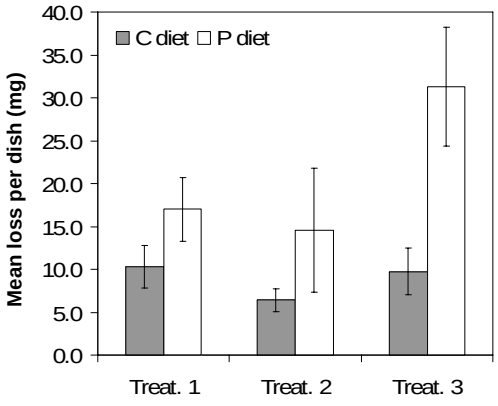


Figure 8. The mean $\pm$ SE weight loss after two days per dish per diet for treatments 1, 2 and 3. The mean $\pm$ SE loss for the isolated dishes in treatment 2 was 10.5 $\pm$ 7.87 mg.

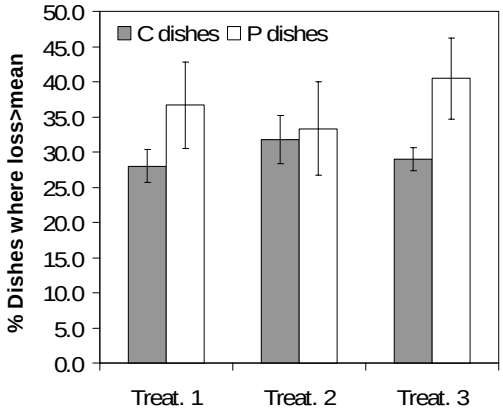


Figure 9. The mean $\pm$ SE number of dishes where the weight loss was higher than the average weight loss per dish per diet. Dishes were counted per trial.

4.2 Foraging patterns

The effect of the different arrangements of the P dishes in each treatment was tested by examining the foraging behaviour of the insects. This was done by looking at the following variables. First, the number of dishes that were visited during day 1 and day 2 of the experiments was analysed (fig 10). In all experiments the locusts visited more dishes on the first day compared to the second day, and visited approximately all dishes over the whole 2 days.

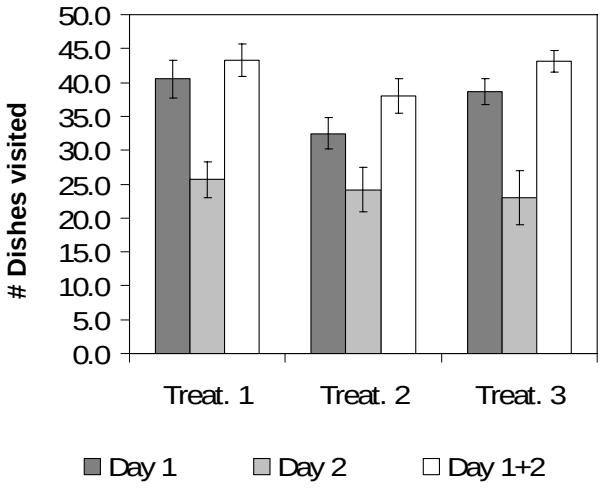


Figure 10. The mean ± SE number of dishes visited on day 1, day 2 and day 1+2 by the locusts from treatments 1, 2 and 3.

Second, the distances between the dishes from which the locusts had eaten the most were analysed. The average distances between the P dish with the highest weight loss and the C dish with the highest, 2nd highest or 3rd highest loss were measured. It was hypothesised that the average distance between highest P loss and highest C loss would be around 30-40 cm or less, and should increase for the distance to the dishes with the 2nd and 3rd highest C loss, if the locusts choose to stay close to the P dishes. In an arena where the maximal distance between two food dishes is 85 cm, this would mean that the locust only uses 1/4th of the total arena for foraging. Figure 11a shows that this is not the case for treatment 1 and 3, but could be true for treatment 2.

Finally the distances between the dishes with the highest and 2nd highest P loss, and between those with the highest and 2nd highest C loss were measured (fig. 11b). Close distances in these measurements would mean that the locusts choose to stay close to the same dishes when foraging for P or C diet. For treatment 1 it was estimated that the distance between the P dishes with the highest and 2nd highest loss would be around 10-20 cm, since they were arranged in a clump where the maximal distance between two P dishes was 20 cm. In treatment 2 the space between the P

dishes with the highest and 2nd highest loss was expected to be 10-20 cm when the locusts choose the clump dishes only, and higher if they also foraged on the isolated dish. The distances for the P dishes in treatment 3 could range between 30 and 85 cm, since all dishes were highly dispersed with maximal in between distances. For treatments 1 and 2, the distances between the C dishes with the highest and 2nd highest loss will represent the actual foraging area the locusts used. For treatment 3 both the distance between the P and the C dishes will represent the locusts foraging area. Figure 11b shows that P dishes with high weight losses in treatment 1 are on average 14 cm apart. This distance was bigger in treatments 2 and 3, where the relevant P dishes are respectively $\pm$ 27 and 54 cm apart. This means that the locusts in treatment 2 also foraged on the isolated dish, and did not always choose the closest P dishes in treatment 3. When looking at the space between the C diets, no difference between treatment 1 and 2 can be detected, suggesting that they have the same sized foraging area. The distances between the C dishes in treatment 3 are very diverse according to the high standard error.

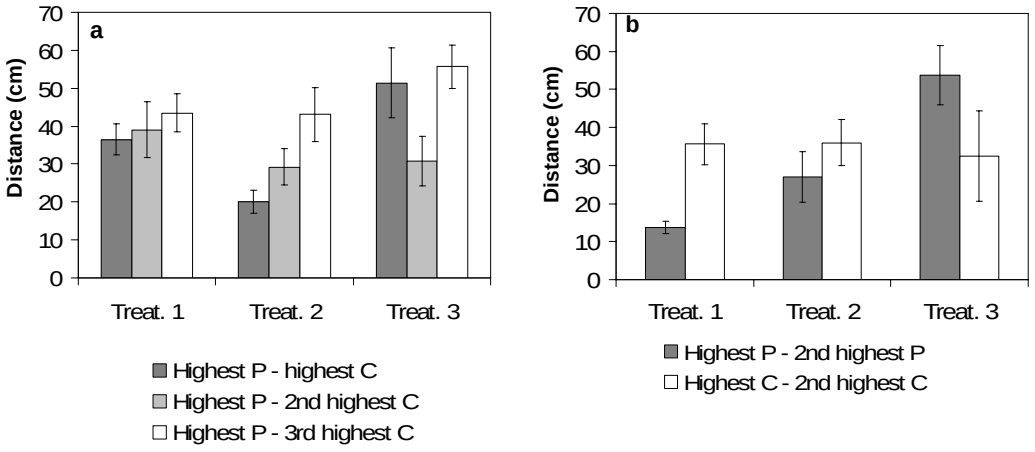


Figure 11. (a) The mean $\pm$ SE distance between the P dish with the highest weight loss and the C dish with the highest, the 2nd highest and the 3rd highest weight loss for treatments 1, 2 and 3. (b) The mean distance between the P dishes with the highest and the 2nd highest weight loss, and between the C dishes with the highest and the 2nd highest weight loss for each of the treatments.

4.3 Associative learning

Finally, the locusts' associative learning abilities were studied. This was done in treatment 1, where the locusts were taken out of the arena on day 3 and left to feed on C diet for 4 h. After this they were reintroduced into the arena, where their behaviour was observed for another 9 h. The P dishes in the arena were replaced by C dishes to make sure that the behaviour of the locusts was based on their associative memory, and not by new encounters and positive feedbacks of available P-dishes. If the locusts learned to associate the visual cues on the walls with the location of the P dishes, their initial behaviour in the arena would be to return to the site where the P dishes had been. To see whether this behaviour occurred, a zone around the previous location of the P dishes was drawn and referred to as P-zone. This zone included the previous P dishes and neighbouring C dishes. The time that the locusts spent in and outside this P-zone was measured for 9 different time periods, with the duration of each period exponentially increasing in length (table 1). Figure 12 shows the average time the locusts spend in both zones for each time period (solid lines). The x-axis represents the actual time, while the y-axis shows the seconds the position of the locust was recorded. The experiments were recorded in time lapse, which means that the position of the locust was recorded for 1 second every 30 seconds. Figure 12 also shows the expected time spend when the locusts would walk around the arena randomly (dotted lines). Since the P-zone is approximately $1/4^{th}$ of the total arena size, it was estimated that the locusts would spend $1/4^{th}$ of each time period in the P-zone and $3/4^{th}$ in the C-zone when foraging randomly. The data from the learning experiment matches the random foraging situation quite well, except for the 3^{rd} (4 min) and the 8^{th} (128 min) time period. These differences are however not significant, so based on this data the conclusion would be that the locusts did not associate the visuals with the location of the protein dishes. However, when looking at the absolute position of the locusts in the arena in the first 2 h (fig. 13) it is clear that they often preferred to sit on perches next to the P-zone.

Table 1. The number of seconds of recording time for each time period and the actual time at the end of each period. Time-lapse recording was used during the experiments, so that the position of the insect was recorded for 1 sec each 30 seconds.

Time Period	Recording time (sec)	Actual Time (min)
1	2	1
2	2	2
3	4	4
4	8	8
5	16	16
6	32	32
7	64	64
8	128	128
9	256	256

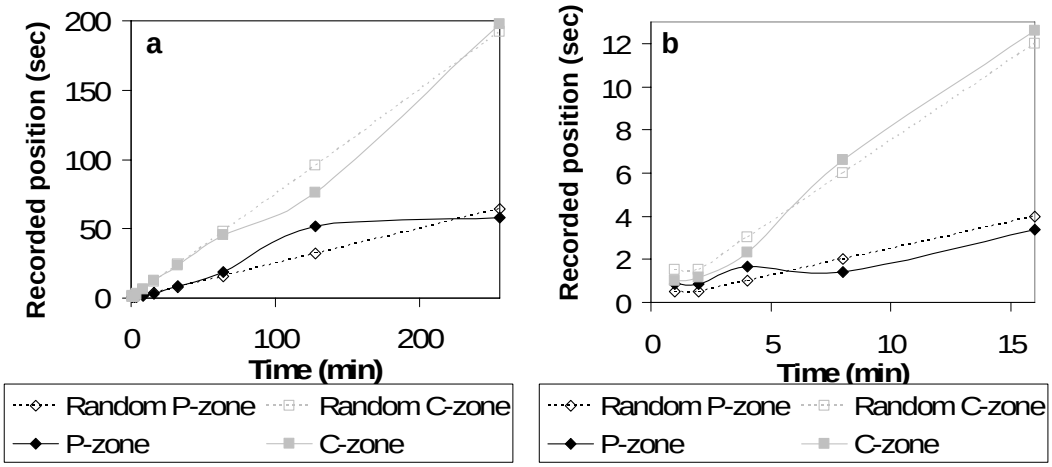


Figure 12. The average position of the locusts in the arena for the time periods 1-9 (a), and 1-5 (b). The x-axis represents the actual time during the experiment, whereas the y-axis shows the number of seconds for which the position of the locusts was recorded.

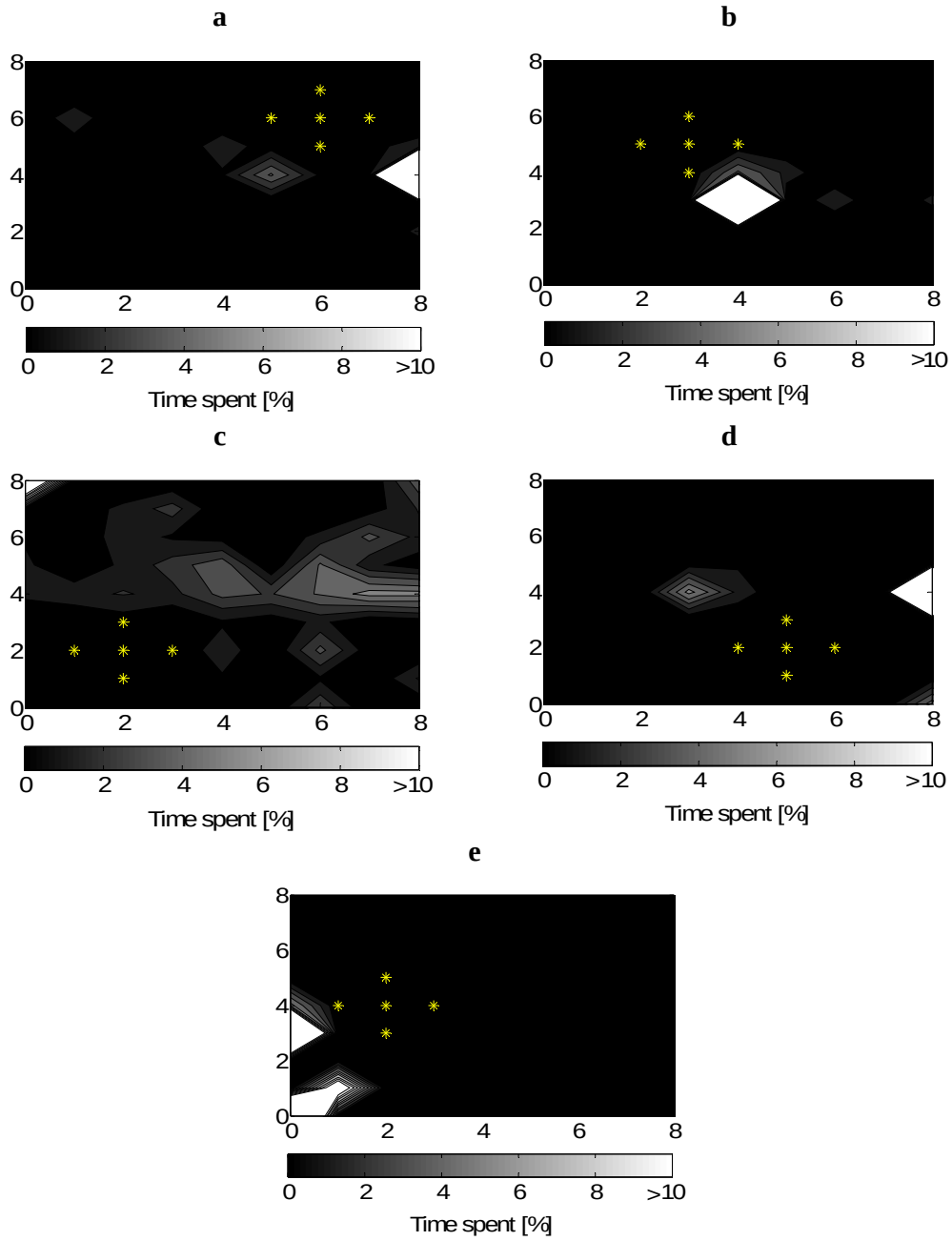


Figure 13. The relative time spent after 2 h by each individual locust on day 3 of experiment 1. The figures a-e represent the arenas of trail 1 (a), 3 (b), 4 (c), 5 (d) and 6 (e). There is no data form the locust in trail 2 because it climbed the wall after 15 s. The x- and the y-axis represent the positions of the dishes in the arena, which are located on (1,1) till (7,7). The location of the P dishes are indicated by the stars (*). Perches are located on (0,0), (0,4), (0,8), (3,4), (4,0), (4,3), (4,5), (4,8), (5,4), (8,0), (8,4) and (8,8). The arrangement of the visuals was the same for each of the arenas, with the blank wall north, the square south, the two vertical stripes west and the three horizontal stripes east.

5. Discussion

5.1 Different P-diet arrangements affect nutrient intake and foraging behaviour

Overall the results indicate that there is not much difference in intake and foraging behaviour between the 3 treatments. Locusts seem to choose the same protein and carbohydrate intake points, have the same size foraging area (although the area for treatment 3 is slightly larger), similar mean loss per P-dish and per C-dish, and have the same portion of P- and C-dishes from which they ate more than average. The locusts in treatment 2 also foraged on both the isolated and the clump P-dishes, which had the same impact on both kinds of dishes. These results indicate that it does not matter which of these strategies (all clumped, one isolated and the rest clumped, or over-dispersed) the plant chooses, the damage caused by herbivorous insects will be the same. Nevertheless, there seems to be a difference for treatment 3. Although it is not significant, locusts in treatment 3 seem to have eaten more protein compared to the other two. This could be due to the number of replicates in the treatments.

The number of replicates in each of the treatments was 6, which is quite low. An increase in replicates could therefore possibly provide prove that treatment 3 is significantly different. Another point is that the locusts from the population at the School of Biological Sciences at University of Sydney could not be used due to problems with the CT room. It was therefore decided to use locusts that were collected from the zoo. Because the first batch was too small for all the trails, a second batch was used, which was picked up later. All this could mean that the locusts used were too different from each other in behaviour and nutrient requirements, making it difficult to draw significant conclusions form the data. The intake for the 3 experiments was also overestimated because the loss per dish could not be determined. This affected the total carbohydrate intake more than the protein intake, since there were more C dishes (44) than P dishes (5). Finally, the animals that were used were only selected by their weight, and not by their sex, what caused a random use of males and females. In treatment 3 nearly only females were used (5 females, 1 male), what could have influenced the data. Treatment 1 consisted of 2 females and 4 males, and in treatment 2 the number was even. In general, it is suggested that the number of males and females should be constant or even in all

treatments, and the number of replicates should increase. Using locusts from the population in our lab could also make a difference. The data from the control group was too different from the treatments to be useful, so it was decided to leave it out. This could be due to the smaller arena in which they were kept, or just because this experiment was carried out later than the 3 treatments.

When looking at the foraging behaviour of the locusts, it is clear that the number of dishes visited is always lower on day 2 compared to day 1 in all of the experiments (fig. 10), indicating that the locusts adapt their behaviour to their environment and choose only a certain part of the arena for foraging. The same change in behaviour was found by Behmer *et al.* (2003), where it was suggested that this change could be due to learning by the animal, or by the fact that the insects rested more next to the P-dish, since a high protein meal has a greater inhibitory influence on the animal.

The area that the locusts used for foraging in each of the treatments can be obtained from the distance between the different P- and C-dishes with the highest weight losses (fig. 11). The locusts in treatments 1 and 2 have approximately the same foraging area size, since the distances between the dishes with the highest weight losses in both treatments range between 20 and 50 cm. The foraging area for treatment 3 however, is slightly bigger, with distances ranging between 30 and 50 cm.

The fact that the locusts adjust their foraging area on the second day suggests that the dimensions and variations within the treatments were meaningful for locusts. It is however not possible to tell whether the locusts in this project select between or within patches, since it was not possible to distinguish between patches within the arena. An experiment in which this is possible would contain different patches build up from 2 or 3 dishes, with the patches being as far away form each other as possible. In addition to this, the diets itself could also be changed. Instead of using a high P and a high C diet, an optimal P/C diet could be used, containing different concentrations of a harmful chemical to make the difference between the palatable and the unpalatable food.

In this project only individual locusts were used, however, in nature locusts can form massive plagues, affecting the plant community in a completely different level. It would therefore be interesting to study the effect of different densities of foraging locusts on the plants and the foraging behaviour of the locusts. For large herbivore mammals it already has been show that higher densities affect the nutrient

intake and foraging behaviour of the individuals. Because of increasing competition between the individuals within the group, it has been suggested that the within-patch selectivity for an individual will decrease as the group size increases, resulting in a higher intake of the less palatable food (Krebs & Davies, 1999; Molvar & Bowyer, 1994; Alm Bergvall *et al*., 2006).

5.2 Associative memory

In the associative learning experiment it was difficult to say whether the locusts had learned to associate the site of the protein dishes with the visual cues on the walls. Figure 12 suggests that the locusts walk around randomly, however, it appears from figure 13, which shows the actual position of the locusts, that the locusts often choose to sit close to the site where the P-dishes had been. Two locusts (fig. 13c and d) clearly do not use associative memory, but walk around randomly. For the other three it could be said that they do not actively search for the P-dishes, but that they do recognize the site where they used to find the P-diet. Probably more replications would give a better result in this experiment.

L. migratoria have been shown to be able to associate the presence certain food sources with different environmental cues, such as odour (Simpson &White, 1990) and visual cues (Raubenheimer & Tucker, 1997). However, these experiments were carried out in relative simple arenas, where the locusts only had two choices. The arena in this experiment could be too complex, or just too big for the locusts. Nevertheless, they seem to restrict their foraging to a certain part of the arena on the second day of the experiments, what indicates that they learn the set up of their environment.

Associative learning is also influenced by the nutritional state the locust is in at the time of learning. This is called state-dependent valuation learning, which means that the feedback of the reward, in this case protein diet, is greater when the animal is deficit for the nutrients (protein) in the reward (Pompilio *et al*., 2006). A locust that is highly protein deficit will therefore have a stronger association of the environmental cues with the presence of protein diet, compared to a locust that just had a protein meal. That means that when the locusts in our experiment find the protein quickly on their first day in the arena they will not be protein deficit, and therefore develop a

weak or, no association between the location of the P-dishes and the visual cues on the arena walls.

Overall, the results suggest that, at least in 3 trails, the locusts learned to associate the location with the visual cues on the walls of the arena. This result is however not significant, suggesting that more replications need to be carried out. Apart from this, locusts need to be protein deficit on their very entry in the arena, so that the encounter with a protein dish will give a strong feedback to the animal. These locusts will then develop a stronger associative memory, what could change their behaviour on the third day.

6. References

Alm Bergvall U., Rautio P., Kesti K., Tuomi J., Leimar O. (2006) *Associational effects of plant defences in relation to within- and between-patch food choice by a mammalian herbivore: neighbour contrast susceptibility and defence.* Oecologia, **147**: p. 253-260.

Atsatt P.R., O'Down D.J. (1976) *Plant defence guilds*. Science, **193**: p. 24-29.

Augustine D.J., Mc Naughton S.J. (1998) *Ungulate effects on the functional species composition of plant communities: herbivore selectivity and plant tolerance.* J. Wildl. Manage, **62**: p. 1165-1183.

Bailey D.W., Gross J.E., Laca E.A. *et al*. (1996) *Mechanisms that result in large herbivore grazing distribution patterns*. J. Range Manage, **49**: 386-400.

Behmer S.T., Cox E., Raubenheimer D., Simpson S.J. (2003) *Food distance and its effect on nutrient balancing in a mobile insect herbivore*. Animal Behaviour, **66**: p. 665-675.

Behmer S.T., Elias D.O., Bernays E.A. (1999) *Post-ingestive feedbacks and associative learning regulate the intake of unsuitable sterols in a generalist grasshopper*. The Journal of Experimental Biology, **202**: p. 739-748.

Behmer S.T., Raubenheimer D., Simpson S.J. (2001) *Frequency-dependent food selection in locusts: a geometric analysis of the role of nutrient balancing.* Animal Behaviour, **61**: p. 995-1005.

Behmer S.T., Simpson S.J., Raubenheimer D. (2002) *Herbivore foraging in chemically heterogeneous environments: nutrients and secondary metabolites*. Ecology, **83**: p. 2489-2501.

Bernays E.A., Wrubel R.P. (1985) *Learning grasshoppers - association of color light intensity with food*. Physiological Entomology, **10**(4): p. 359-369.

Champagne D.E., Bernays E.A. (1991) *Phytosterol unsuitability as a factor mediating food aversion learning in the grasshopper* Schistocerca americana. Physiological Entomology **16**: p. 391-400.

Distel R.A., Laca E.A., Griggs T.C., *et al*. (1995) *Patch selection by cattle: maximization of intake rate in horizontal heterogeneous pastures.* Appl. Anim. Behav. Sci., **45**: 11-21.

Goldsmith C.M., Hepburn H.R., Mitchell D. (1978) *Retention of an associating learning task after metamorphosis in* Locusta migratoria migratorioides. Journal of Insect Physiology, **24**: p. 737-741.

Hjältén J., Danell K., Lundberg P. (1993) *Herbivore avoidance by association: vole and hare utilization of woody plants*. Oikos, **68**: p. 125-131.

Hokkanen H.M.T. (1991) *Trap cropping in pest-management*. Annu. Rev. Entomol., **36**: p. 119-138.

Holliday J.L., Holliday N.J. (1995) *Changes in learning ability and mechanisms during development of grasshopper nymphs*, Melanoplus bivattatus. Physiological Entomology, **20**(2): p. 109-116.

Kotliar N.B., Wiens J.A. (1990) *Multiple scales of patchiness and patch structure – a hierarchical framework for the study of heterogeneity*. Oikos, **59**: p. 253-260.

Milchunas D.G., Noy-Meir I. (2002) *Grazing refuges, external avoidance of herbivory and plant diversity*. Oikos, **99**(1): p. 113-130.

Raubenheimer D. (1992) *Tannic acid, protein, and digestible carbohydrate: dietary imbalance and nutritional compensation in the African migratory locust*. Ecology, **73**: 879-890.

Raubenheimer D., Blackshaw J. (1994) *Locusts learn to associate visual stimuli with drinking*. Journal of Insect Behavior, **7**(4): 569-575.

Raubenheimer D., Simpson S.J. (1999) *Intergrating nutrition: a geometrical approach*. Entomologia Experimentalis et Applicata, **91**(1): p. 67-82.

Raubenheimer D., Simpson S.J. (2002) *Unravelling the tangle of nutritional complexity*. Wissenschaftskolleg zu Berlin, Jahrbuch 2002/2003: p. 275-294.

Raubenheimer D., Simpson S.J. (2003) *Nutrient balancing in grasshoppers: behavioural and physiological correlates of dietary breadth*. The Journal of Experimental Biology **206**: p. 1669-1681.

Raubenheimer D., Simpson S.J. (2004a) *Organismal stoichiometry: quantifying non-independence among food components*. Ecology, **85**(5): p. 1203-1216.

Simpson S.J., Sibly R.M., Lee K.P., Behmer S.T., Raubenheimer D. (2004) *Optimal foraging when regulating intake of multiple nutrients*. Animal Behaviour, **68**: p. 1299-1311.

Raubenheimer D., Tucker D. (1997) *Associative learning by locusts: pairing visual cues with consumption of protein and carbohydrate*. Animal Behaviour, **54**: p. 1449-1459.

Roguet C., Dumont B., Prache S. (1998) *Selection and use of feeding sites and feeding stations by herbivores: a review*. Ann. Zootechnie., **47**., 225-244.

Searle K.R., Thompson Hobbs N., Shipley L.A. (2005) *Should I stay or should I go? Patch departuredecisions by herbivores at multiple scales*. Oikos, **111**(3): p. 417-424.

Senft R.L., Coughenour M.B., Bailey D.W. *et al*. (1987) *Large herbivore foraging and ecological hierarchies*. Bioscience, **37**: p. 789-799.

Simpson S.J., Abisgold J.D. (1985) *Compensation by locusts for changes in dietary nutriens: behavioural mechanisms*. Physiological Entomology, **10**: p. 443-452.

Simpson S.J., Raubenheimer D. (2001) *The geometrical analysis of nutrient-allochemical interactions: a case study using locusts*. Ecology, **82**: p. 422-439.

Simpson S.J., Raubenheimer D., Behmer S.T., Whitworth A., Wright G.A. (2002) *A comparison of nutritional regulation in solitarious- and gregarious-phase nymphs of the desert locust* Schistocerca gergaria. The Journal of Experimental Biology, **205**: p. 121-129.

Simpson S.J., White P.R. (1990) *Associative learning and locust feeding: evidence for a 'learned hunger' for protein*. Animal Behaviour, **40**: p. 506-513.

Visås H.J., Sæther B.E. (1987) *Interactions between a generalist herbivore, the moose* Alces alces, *and its food recources: an experimental study of winter foraging behaviour in relation to browse availability*. J. Anim. Ecol., **56**: 509-520.

Zee B. van der, Behmer S.T., Simpson S.J. (2002) *Food mixing strategies in the desert locust: effects of phase, distance between foods, and foods nutrient content*. Entomologia Experimentalis et Applicata, **103**: p. 227-237.

Appendix A

Preparation of Artificial Diets

1. Establish which diets and what amounts are likely to be required for duration of experiment
   (artificial diets.xls: fill in the **bold** numbers % protein, % carbohydrate and the total amount). Print spreadsheet = Appendix 1.
2. Check first to see that all the ingredients are available (Appendix 3).
3. Collect sufficient 3 litre glass beakers (one for each diet) from shelf, ensure they are clean.
4. Label each beaker clearly with correct diet formula.

Mixing Formula

Day One

1. Make sure balance is set at zero and clean.
2. Place beaker on balance and re-zero.
3. Measure out required amount of cellulose using spatula for each diet (e.g. 5 diets - 5 amounts of cellulose). Place each amount in relevant beaker. It is advisable to 'tick off ingredients on Appendix 1 as you proceed.
4. Do likewise with casein and stir well in fume cupboard.
5. Measure out required amount of cholesterol (kept in fridge) in beaker (large enough to take the chloroform). Using 1 m1 syringe (without needle) (or autopipetter with the end of the tip removed) add to the cholesterol the linoleic acid and mix in carefully.
6. Take each beaker to the fume cupboard together with each cholesterol/linoleic acid weighing boat. Wearing mask and gloves remove chloroform (not redistilled) from the chemical store.
7. Pour required amount of chloroform into a measuring cyclinder and introduce to the cholesterol/linoleic acid mixture. Make sure it is completely dissolved before adding to the beaker with the previously measured amounts of cellulose and casein. Repeat for each diet.
8. Mix thoroughly with glass stirring rod. Continue to stir throughout the day to ensure lumps or concentrates do not form.
9. Leave in the fume cupboard 24 h or until the chloroform has completely evaporated.

Day Two

1. Add Wesson salts, Sucrose, Dextrin, Peptone, Albumen and Vitamin C to relevant diets (remembering to tick off ingredients as you proceed). Stir thoroughly.
2. Dissolve vitamin mix[1] (found in freezer) in 20% Ethanol (20% absolute ethanol 80% de-ionized water).

[1] See Ingredients for recipe

NB It is advisable to thoroughly dissolve the vitamin mix in required amount of alcohol and then top up with de-ionized water (avoid creating a sediment).

3. Pour into dry mix and stir thoroughly until homogeneous.
   NB for high protein diets (28% *or higher) mix up approximately half the amount of 20% EtoH quoted on Appendix* 1; *otherwise the diet produced will prove to be too hard.*
4. Place aluminum foil on a white tray and label clearly diet formula (one tray per diet).
5. Mix each diet in food processor with mixer blade on 'whisk' for about 1-2 min.
6. Pour diet onto relevant tray and spread evenly and thinly as possible breaking up any lumps.
   Repeat for all diets

Place trays in oven at 30°C for 24 h.

Day 3

1. Place each diet in food processor again for about 30 sec's to break up crust etc.
2. Re-deposit on original tray and return to oven for further drying.

Day 4

1. Diet should now be dried. Grind diet if necessary and place in sealed box (clearly labeled) in-20^{o}C freezer until required. (Diets must be kept in freezer).

Website

Fill in required amount and ratio of protein and carbohydrate in spreadsheet "artificial diets.xls" and print. Spreadsheet "artificial diets.xls" is located either on the computer attached to the scales or under the SimpsonLab folder on \\Wallace\usersa08\SimpsonLab\Artificial diet\ artificial diets.xls.

Ingredients

1. See table for ingredients.
2. Re-zero balance between measuring each ingredient.
3. Add each ingredient to a mortar.
4. Mix thoroughly in mortar and pestle until orange-yellow in colour.
5. Keep in sealed container with desiccant in freezer.

Vitamin Mix

Thiamine	Sigma:	T4625 (5g)	0.075g
Riboflavin		R4500 (5g)	0.075g
Nicotinic acid		N4126	0.300g
Pyridoxine		P9755 (10g)	0.075g
Folic acid		F7876 (1g)	0.075g
Meso-inositol		I5125	0.750g
Calcium pantothenate		P2250	0.150g
p-aminobenzoic acid		A9878 (5g)	0.075g
Choline chloride		C1879	3.750g

Biotin	B4501 (1g)	0.003g
Total		***5.328g***

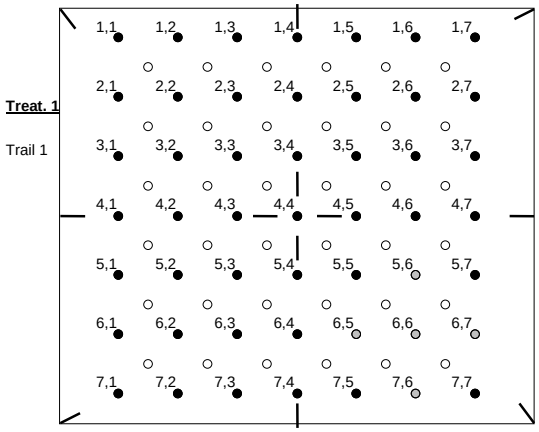
Artificial diet chemicals

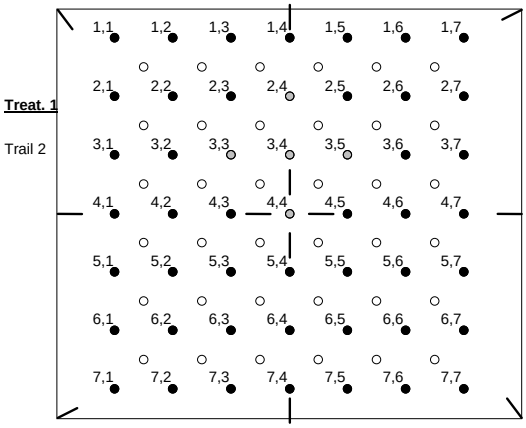
Cellulose	Sigma:	C8002
Casein		C3400
Dextrin		D2131
Linoleic acid (approx. 60%)		L1626
Cholesterol		C3292
Wesson's salts		W1375
L-ascorbic acid		25564
Peptone	Oxoid	LP0037
Sucrose	AnalaR BDH	10274

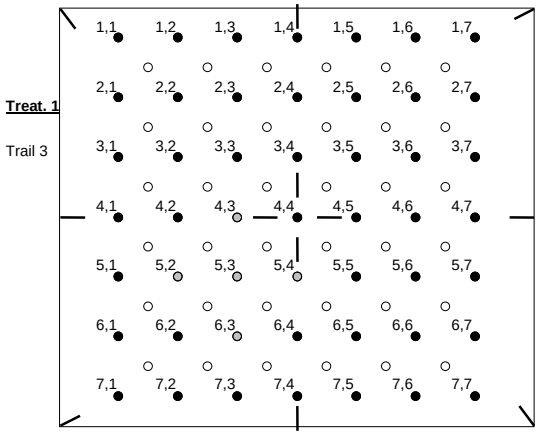
Albumen – for Australians, Pace farms (due to quarantine restrictions (avian influenza) egg products need to be sourced within each country, Sigma preferred supplier)

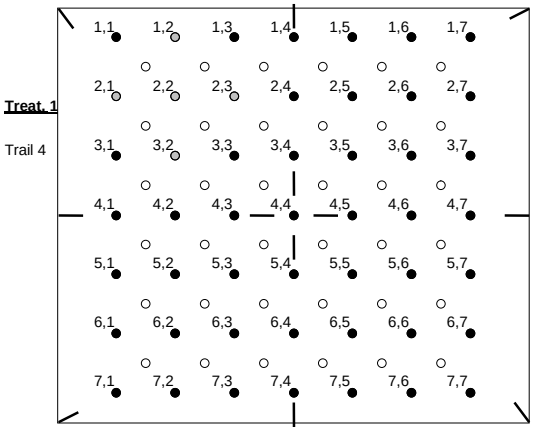
Appendix B

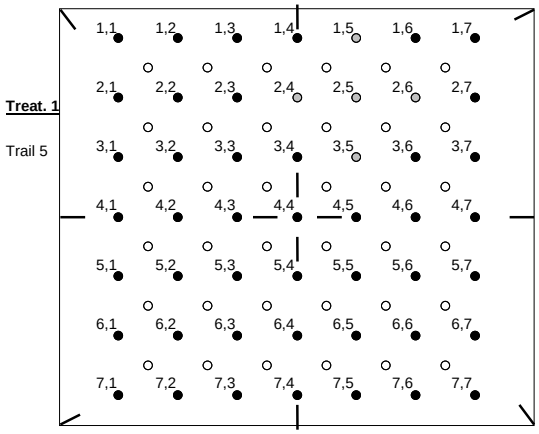
Treatment 1

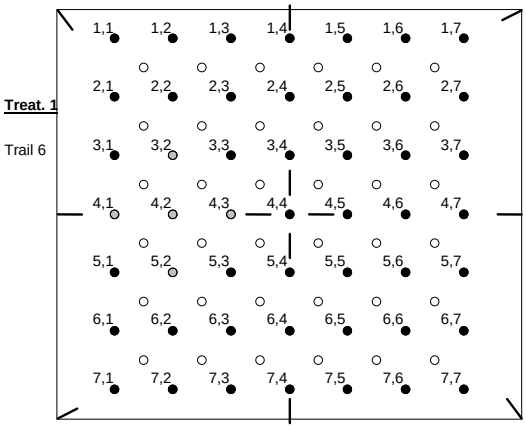




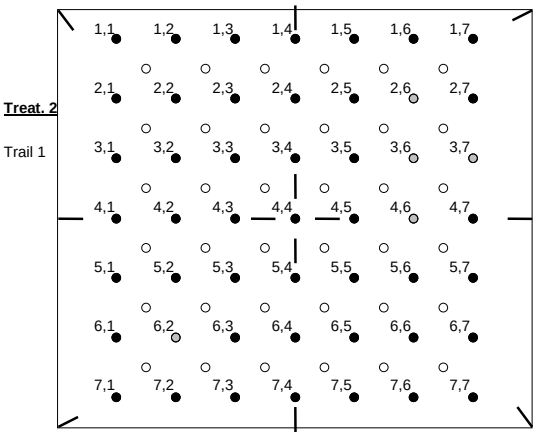


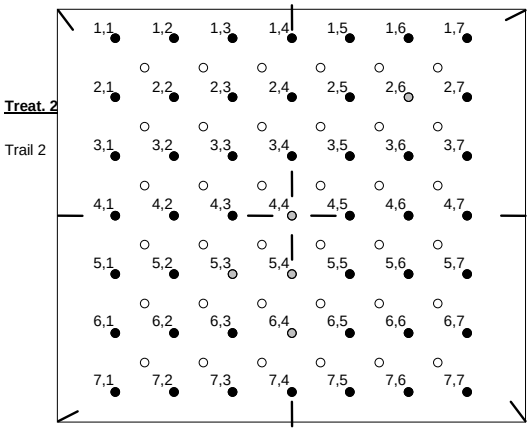






Treatment 2


Treat. 2
Trail 1
1,1 1,2 1,3 1,4 1,5 1,6 1,7
2,1 2,2 2,3 2,4 2,5 2,6 2,7
3,1 3,2 3,3 3,4 3,5 3,6 3,7
4,1 4,2 4,3 4,4 4,5 4,6 4,7
5,1 5,2 5,3 5,4 5,5 5,6 5,7
6,1 6,2 6,3 6,4 6,5 6,6 6,7
7,1 7,2 7,3 7,4 7,5 7,6 7,7


Treat. 2
Trail 2
1,1 1,2 1,3 1,4 1,5 1,6 1,7
2,1 2,2 2,3 2,4 2,5 2,6 2,7
3,1 3,2 3,3 3,4 3,5 3,6 3,7
4,1 4,2 4,3 4,4 4,5 4,6 4,7
5,1 5,2 5,3 5,4 5,5 5,6 5,7
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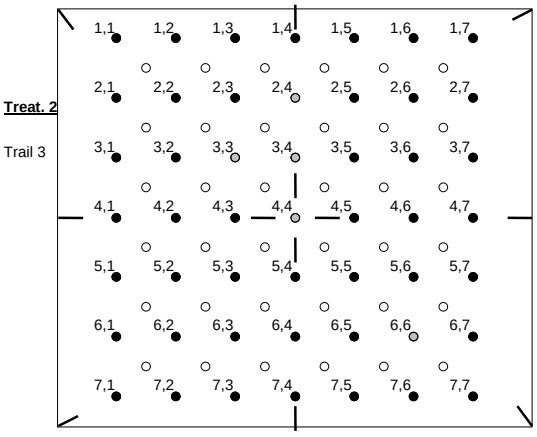
7,1 7,2 7,3 7,4 7,5 7,6 7,7


Treat. 2
Trail 3
1,1 1,2 1,3 1,4 1,5 1,6 1,7
2,1 2,2 2,3 2,4 2,5 2,6 2,7
3,1 3,2 3,3 3,4 3,5 3,6 3,7
4,1 4,2 4,3 4,4 4,5 4,6 4,7
5,1 5,2 5,3 5,4 5,5 5,6 5,7
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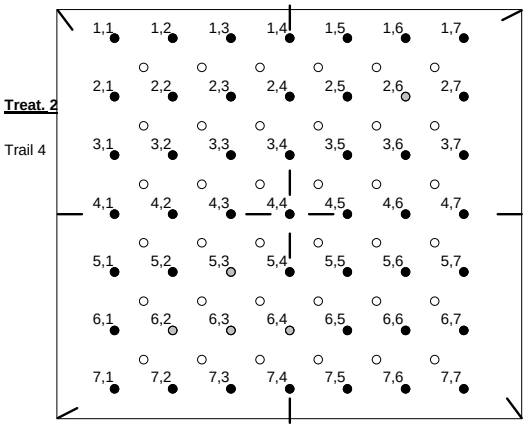
7,1 7,2 7,3 7,4 7,5 7,6 7,7


Treat. 2
Trail 4
1,1 1,2 1,3 1,4 1,5 1,6 1,7
2,1 2,2 2,3 2,4 2,5 2,6 2,7
3,1 3,2 3,3 3,4 3,5 3,6 3,7
4,1 4,2 4,3 4,4 4,5 4,6 4,7
5,1 5,2 5,3 5,4 5,5 5,6 5,7
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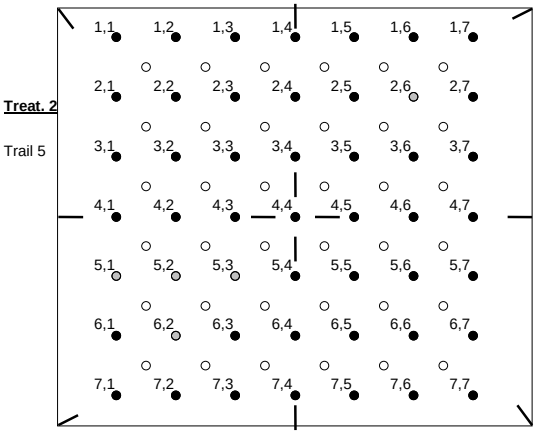
7,1 7,2 7,3 7,4 7,5 7,6 7,7


Treat. 2
Trail 5
1,1 1,2 1,3 1,4 1,5 1,6 1,7
2,1 2,2 2,3 2,4 2,5 2,6 2,7
3,1 3,2 3,3 3,4 3,5 3,6 3,7
4,1 4,2 4,3 4,4 4,5 4,6 4,7
5,1 5,2 5,3 5,4 5,5 5,6 5,7
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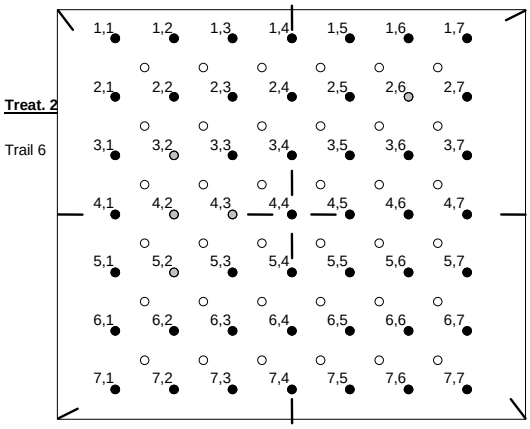
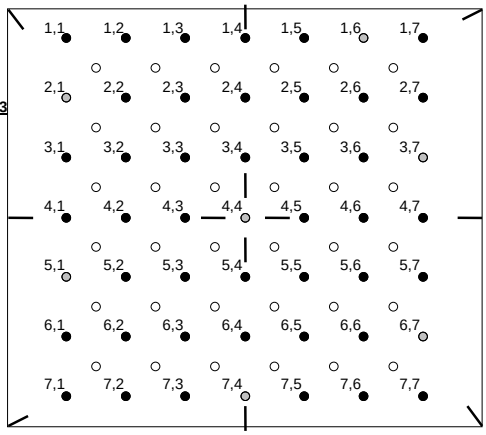
7,1 7,2 7,3 7,4 7,5 7,6 7,7


Treat. 2
Trail 6
1,1 1,2 1,3 1,4 1,5 1,6 1,7
2,1 2,2 2,3 2,4 2,5 2,6 2,7
3,1 3,2 3,3 3,4 3,5 3,6 3,7
4,1 4,2 4,3 4,4 4,5 4,6 4,7
5,1 5,2 5,3 5,4 5,5 5,6 5,7
6,1 6,2 6,3 6,4 6,5 6,6 6,7
7,1 7,2 7,3 7,4 7,5 7,6 7,7

Treatment 3

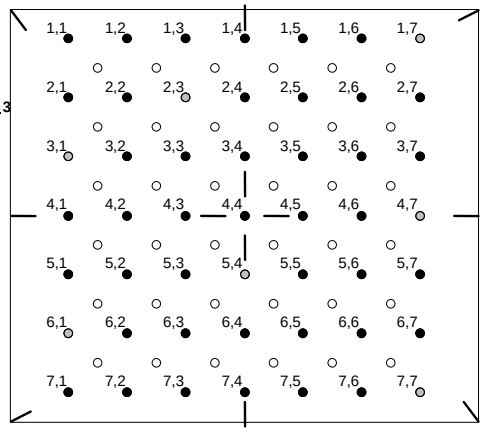
Treat. 3

Trail 1



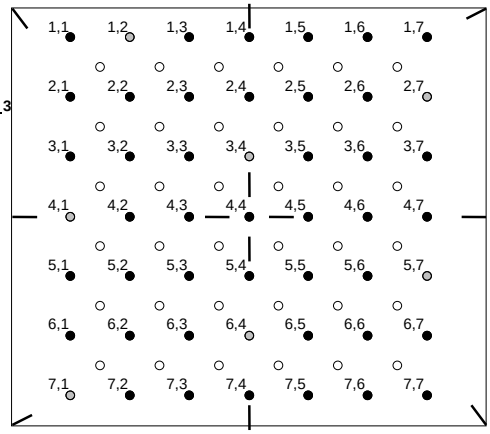
Treat. 3

Trail 2



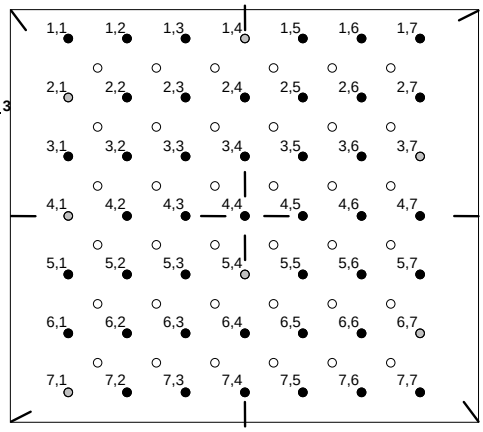
Treat. 3

Trail 3



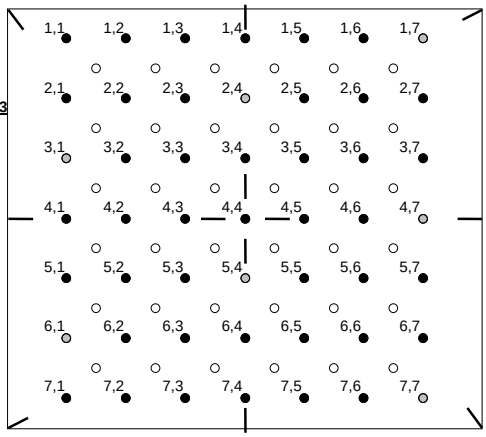
Treat. 3

Trail 4



Treat. 3

Trail 5



Exp. 3

Trail 6