OPTIMISATION OF THE REARING METHOD FOR THE TWO SPOT LADYBIRD (ADALIA BIPUNCTATA)

Diet effect on oviposition and fertility rate

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Mating of a 2 spot typica couple


Oviposition by a 2 spot melanic form


Larva


Pupa


Adult sucking an aphid

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PREFACE

The experiments described in this report were conducted at Entocare BV, as part of my internship in entomology. This practical period is compulsory at Wageningen University to finalise the study programme of my MSc in Organic Agriculture.

Being specifically interested in the biological control of agricultural pests, my choice of performing the internship in this company was a result of wanting to combine scientific research with the commercial aspects of the production of natural enemies.

I would like to acknowledge Entocare for the opportunity to conduct my internship at their facilities and getting acquainted with the daily aspects of the production of biological products (which are not part of this report).

A special thanks to Dr. Roel van der Meiracker, for the help in elaborating all the experimentals designs, to Miriam, for the extensive practical explanations about all aspects of the rearing system for *Adalia bipunctata* and finally to Dr. Peter de Jong, for the guidance throughout different aspects of the internship.

1. INTRODUCTION

1.1. A small overview of ladybirds' life history

Ladybirds overwinter in shelters, often congregating in groups, and wait five to eight months until their next meal. Adults will become active and start feeding and mating as soon as climatic conditions are good (and also as soon as prey is available). The cues, which help each species of ladybirds to trigger to become active, are largely unknown but temperature and day length are definitely important (Hodek, 1973).

After feeding (first thing they do after they wake up), they will look for a mate: there is no long-distance attraction between sexes (no pheromones or sound involved) and males just seem to find females by bumping into them (probably there is some short-range visual attraction), and their first reaction when encountering another ladybird is to climb onto its back. If all goes well, mating will last from one to eight hours. Most species are highly promiscuous and in the two spot ladybirds, repeated matings are necessary to maintain a high level of egg fertility. A female will mate on average 20 times during the spring reproductive period (Majerus, 1994).

Females will lay eggs (20-50 a day) in batches (the maximum record for the two spot is 55 eggs in a single batch) that become grey just before the larvae hatch (Majerus, 1994). All the larvae of a particular batch of eggs will hatch more or less at the same time (depending on the climate but generally 4-8 days after oviposition took place). Duration of larval stages varies with weather conditions and food abundance but the total duration of the larval stage is typically 3-4 weeks in predatory species. When a fully-grown larvae stops feeding, it is ready to pupate**.** Twenty four to forty eight hours before pupating, the larvae will become immobile and attach itself to a substrate. A week or so after pupation (depending on temperature), an adult will emerge. Adult stages of the two spot ladybirds will become reproductively mature approximately only 1 week, after emergence (Majerus, 1994 and Blackman, 1974).

Ladybirds have long been used to control aphid pests (both as larvae and adults) as they are their most common natural enemy (Blackman, 1974) and even though they are highly polyphagous, aphids are definitely their favourite food in the wild. When aphid densities decrease, they will feed on other available insects and even pollen. A lot of discussion has arisen on the efficiency of these coccinellidae as biological control agents of aphids and the dynamics of predator-prey populations have been extensively studied. Even though more research is needed on this topic, these beetles are being more commonly used by farmers who reject chemicals and their production by private companies has increased over the years. But like for the production of every biological control agent, successful rearing techniques are necessary to rear larvae and adults of ladybirds.

1.2. Overview of rearing two spot ladybirds

Most companies that produce natural enemies and biological products to fight agricultural pests in a sustainable way, tend to keep their rearing systems quite confidential. However, scientific literature is available on the different climatic conditions and prey preference of adults and larvae (for instance) and how that affects larval development and adult mortality.

Many different authors have found that larval development, oviposition, fecundity and total developmental time are directly influenced by the prey quality (Blackman, 1967b; Hodek & Hodek, 1996; Mills, 1981; Özder & Saglam, 2003). Thus, even though *Ephestia* eggs are currently being used (and quite efficiently) by Entocare, complementing the diet with the ladybirds' natural prey (many different aphid species) seems logical (the question remains which is more suitable in economical terms: buying the *Ephestia* eggs or rearing/buying aphids. However, that was not a question to be answered in this report).

Kalushkov (1998) tested 10 aphid species as prey for the two spot ladybird and found six new species that are essential preys (*Euceraphis betulae*, *Cavariella konoi*, *Liosomaphis berberidis*, *Acyrthosiphon ignotum*, *Aphis farinosa* and *Macrosiphoniella artemisiae*). Essential preys being those that result in different developmental rates, fecundity and survival, whilst presenting different degrees of favourability (Hodek & Honek, 1996). *M. artemisiae* and *A. ignotum* were very profitable food (according to rate of larval development, larval mortality and adult fresh weight), in the laboratory even though they do not occur in the field together with the two spot ladybird.

Also, Blackman (1965) had already addressed the suitability of different aphid species for the two spot reared in the laboratory (at 20 °C and 16:8 light regime) and found that *Aulacorthum circumflexum* was the aphid species that resulted in the lowest developmental time (9.5 days), a high mortality during larval development (16.7%) and a not so high adult weight at emergence (11.9 mg). For the aphid species used in the experiments described in this report (*A. pisum*), the mean developmental time was only a little different (10.8 days), larval mortality was much lower (13.9%) and adult weight a bit better (12.6 mg). Blackman (1965) also found that the worst aphid species was A*phis sambuci*, which resulted in 13.4 days of developmental time, 25% larval mortality and 8 mg of adult weight at emergence.

From the research done so far, it is clear that temperature, humidity and light regime are important factors to consider in a mass rearing system for they affect directly larval development, oviposition and adult weight. Entocare, however, seems to have that under control. Nevertheless, ladybirds are peculiar when it comes to hygiene and it seems they will eat more and lay more eggs, if the units where they are reared are kept under good conditions.

As for the food factor, it is also clear that providing different aphid species also affects the parameters mentioned before. Ladybirds are not spoiled insects and they will eat different aphids, according to their availability. The point is how easy and costly it is to rear those species, considering the results they offer. Considering this, the species being currently provided at Entocare seems to be a good choice.

2. THE RESEARCH QUESTION AND EXPERIMENTAL DESIGN

The main objective of the experiments described in this report was to improve the existing rearing method of *Adalia bipunctata* used by Entocare but this current method is not exactly the same as the one used before. However, due to the confidentiality of their rearing methods, that one and the changes it went through until it became the current method, will not be described in this report.

2.1 Current Rearing Method

The method being used this year has proved to be highly laborious and not so efficient:

- adults are being placed in groups of 50 individuals in 3l plastic boxes covered with glass
- diet provided consists of: *Ephestia* eggs, provided in small plastic lids; honey diluted in water (2:1), brushed on top of cotton pieces that were placed in small plastic lids, and the former refreshed when necessary; live or frozen *Acyrthosiphon pisum* aphids (amounts given vary with their availability)
- beetles are fed every four days
- pieces of burlap are placed in the boxes as an oviposition substrate
- rearing conditions are 25 °C temperature and 35 % relative humidity, in the rearing room (these values are certainly higher, inside the rearing units)

2.2. Rearing Problems

This method has proved in the past to have a main bottleneck: rearing the aphids is quite laborious and not always successful (as it is quite easy to have aphid parasitoids showing up in the rearing units).

But there are other questions regarding this rearing method that have not helped in achieving a successful rearing: the amount of aphids and *Ephestia* eggs per adult and day, necessary for the females to lay the maximum number of eggs is not known; the current fecundity per female or cage is not known, as well as the maximum number possible of laid eggs; the groups are being set up without sexing the beetles first (thus, there are no guarantees that successful matings will occur); the general hygienic conditions are not so good; as the whole rearing system is quite laborious, there is not enough time to keep the cages always in the best condition.

Because the main goal of Entocare is to increase the number of fertile eggs (as the final product to sell are larvae and not eggs) and thus scale up the rearing, the point of these experiments was to find out how that could be achieved. The possibilities are either to increase the total number of adults and deal with the increase in practical work that this implies, or to change the diet in order to achieve higher oviposition and fertility rates.

2.3. Possible Improvements

Thus, to help improve this rearing method, these experiments were planned with the purpose of discovering what is the real effect of different diets on the oviposition and fertility of adult two spot ladybirds *Adalia bipunctata*, reared by Entocare.

2.4. Approach

In order to do this, a decision was made of starting by testing individual couples under different treatments: a control group (basic diet of *Ephestia* eggs and honey) and two treatments, to which either pollen or aphids were given. Because Entocare also uses beetles that have been in diapause for some weeks in their current rearing, these were also included in this experiment (and hence their oviposition and fertility rates compared to the ones of beetles that have been reared continuously).

Because the results of this experiment proved that females do not necessarily need aphids to lay fertile eggs and this can be achieved by providing pollen or even just the basic diet, the next experiment was conducted keeping the beetles in groups (and thus the effect of competition for food and mates could be detected). So, the second experiment was conducted using groups of 40 adults, to which either pollen or aphids were provided too.

Regardless of the results of these experiments, their setup was not really applicable for a serious mass rearing (producing larvae using individual couples or even 40 adults groups is definitely too laborious for Entocare), and thus the third experiment served that purpose: discover the maximum oviposition and fertility rate of females kept in 500 adults groups, and how diet affected that. The next table shows clearly the research objective of each of these experiments.

Table 2.1. Research objective of each experiment

Experiment	Research question	Results show effect of
Individual couples	What is the maximum number of laid and fertile eggs under optimal conditions (no competition for food or mates)?	Different diets on maximum oviposition and fertile period
40 adults groups	What is the maximum number of laid and fertile eggs when beetles are kept in groups?	Pollen or aphids, on oviposition and fertility rate of bigger groups
500 adults groups	What is the maximum number of laid and fertile eggs when beetles are kept in mass rearing cages?	Pollen and frozen aphids, on oviposition and fertility rate of mass rearing units

3. THE EXPERIMENTS

3.1. General Setup

Three different experiments were conducted at Entocare, in order to test the diet effect on the oviposition and fertility rate of *Adalia bipunctata* adults, kept under different experimental setups: couples in individual containers, 40 adults groups in small boxes and 500 adults groups in bigger cages. Rearing conditions for every experiment were 25 °C temperature and 35 % relative humidity.

The basic food provided to all treatments was *Ephestia* eggs and honey and the main difference between the treatments was between providing pollen or aphids.

Considering the fact that all of these experiments were conducted with the specific objective of finding the maximum number of laid eggs under optimal conditions (providing more and better food than believed to be necessary), and not with the objective of finding significant differences between the treatments, the number of replicates used was rather low and thus no statistical analysis of results is conducted in this report.

The following sections further explain the specific setups and methods used in each of the three experiments but the next table gives a general idea of the differences between them:

Table 3.1. General information on the setup of all experiments

<table>
<tr><th>Experiment</th><th>Number of treatments</th><th>Diet</th><th>Adults</th><th>Extras</th></tr>
<tr><td rowspan="3">Individual couples</td><td rowspan="3">6</td><td>Basic</td><td rowspan="3">5 couples per treatment</td><td rowspan="3">All treatments were repeated using beetles that had been in diapause. Couples kept in individual small containers.</td></tr>
<tr><td>Basic + pollen</td></tr>
<tr><td>Basic + aphids</td></tr>
<tr><td rowspan="2">40 adults groups</td><td rowspan="2">2</td><td>Basic + pollen</td><td rowspan="2">40 adults per treatment</td><td rowspan="2">All treatments were repeated once. Each group kept in closed boxes.</td></tr>
<tr><td>Basic + aphids</td></tr>
<tr><td rowspan="3">500 adults groups</td><td rowspan="3">3</td><td>Basic + pollen + aphids 1x week</td><td rowspan="3">500 adults per treatment</td><td rowspan="3">None of the treatments was repeated. Each group kept in cages.</td></tr>
<tr><td>Basic + aphids 3x week</td></tr>
<tr><td>Basic + aphids 1x week</td></tr>
</table>

3.2. Individual couples

Five *Adalia bipunctata* couples were kept in individual plastic containers of 0.5l, per treatment (round, with a ventilated lid). A basic diet of *Ephestia* eggs (10 mg/couple/day) and honey (ad libitum) was provided to all treatments three times per week. Treatment 1 consisted of the control group (basic diet) whilst treatment 2 was also given pollen (ad libitum) and treatment 3 live adult aphids (10 *Acyrthosiphun pisum* per couple, three times per week as well), as shown in table 3.2 and appendix V:

Table 3.2. Experimental setup of the individual couples experiment

<table>
<tr><th>Treatment number</th><th>Ladybirds</th><th>Food</th><th>Ladybird condition</th><th>Food provided as</th></tr>
<tr><td>1</td><td>5 couples</td><td>Ephestia + honey</td><td rowspan="3">Continuous rearing</td><td>Ephestia spread on bottom of the container, honey on a wet cotton dish inside a small plastic lid</td></tr>
<tr><td>2</td><td>5 couples</td><td>Ephestia + honey + pollen</td><td>Both Ephestia and pollen spread on bottom of container, honey on a wet cotton dish inside a small plastic lid</td></tr>
<tr><td>3</td><td>5 couples</td><td>Ephestia + honey + aphids</td><td>Ephestia spread on bottom of box, honey on a wet cotton dish inside a small plastic lid, aphids released into box</td></tr>
<tr><td>4</td><td>5 couples</td><td>Ephestia + honey</td><td rowspan="3">Diapause</td><td>Like in treatment 1</td></tr>
<tr><td>5</td><td>5 couples</td><td>Ephestia + honey + pollen</td><td>Like in treatment 2</td></tr>
<tr><td>6</td><td>5 couples</td><td>Ephestia + honey + aphids</td><td>Like in treatment 3</td></tr>
</table>

The ladybirds used in this experiment came from the continuous rearing at Entocare (larvae fed on *Ephestia* and their parents on *Ephestia*, honey and aphids; rearing conditions 25 °C and 35% RH) and their developmental cycle can be seen in appendix I. All three treatments were repeated using adults that had been kept in diapause (at 8 °C) for about 5 weeks (Appendix I).

Adults were sexed soon after emergence and the couples set up together in the containers and fed according to the treatments. One piece (5 x 5cm) of burlap was placed in the containers to stimulate oviposition, removed every day (except in the weekends) and eggs on it and around the container counted and kept separately for posterior counting of hatched larvae. Containers were changed at least once a week to ensure a highly hygienic condition.

The food provided demanded some special attention:

The *Ephestia* eggs were kept in the freezer (-18 °C) and placed in the fridge (+13 °C) one day before usage and the amounts provided to each couple were always slightly higher than the required 10 mg/couple/day. The amount of *Ephestia* eggs was calculated on the basis of practical knowledge that an adult ladybird will consume 5 mg per day, when no other type of food is provided. This figure is the result of several trials done on ladybirds' diets.

The honey was provided diluted in water in a proportion of 1:2 (honey/water) and given *ad libitum* every time the couples were fed. The cotton where the honey was applied in was changed at least once a week.

The pollen provided was regular bee pollen in the form of grains, stored in the freezer (-18 °C) and removed a few hours before the beetles were fed. The pollen grains were always finely smashed in a mortar to achieve the softest texture possible.

The aphids came from the greenhouse rearing of Entocare (reared on bean plants) and provided always fresh and in the adult stage (4^{th} instar when not enough adults were available).

After 26 days, the experiment was terminated (see appendix II) and the best and worst couples (the ones that had laid the highest and the lowest total number of eggs) of each treatment were kept for another 18 days, just in order to see until when the females would lay eggs, when fed with these different diets (thus, the experiment lasted for a total of 44 days).

3.3. 40 adults groups

In this experiment, 40 adult *Adalia bipunctata* beetles (in a 50:50 proportion of males and females) were kept in plastic boxes of 3l, per treatment (boxes closed with a glass plate on top). A basic diet of *Ephestia* eggs (5 mg/beetle/day) and honey (*ad libitum*) was provided to all treatments three times per week. Treatment 1 consisted of the basic diet plus frozen adult aphids (*Acyrthosiphon pisum*) whilst treatment 2 was only given pollen (*ad libitum*), as shown in the next table and in appendix VI:

Table 3.3. Experimental setup of the 40 adults groups experiment

Treatment	Ladybirds	Food	Food provided as
1 & 2	40 per treatment	*Ephestia* + honey + aphids	*Ephestia* inside small lids on the bottom of box, honey on a wet cotton dish inside a small lid and aphids released into box
3 & 4		*Ephestia* + honey + pollen	Both *Ephestia* and pollen inside small lids on the bottom of box, honey on a wet cotton dish inside a small lid

Two replicates of each treatment were done at the same time, with different beetles. Procedures regarding the ladybirds used, the basic diet, pollen and aphids (and thus amounts provided) were all like in the experiment described in the previous section. When not enough living aphids were available, frozen ones were provided (numbers given varied according to availability of the aphids). Three pieces of burlap (8 x 8cm) were placed in the boxes, removed every day and eggs on it and around the box counted and kept separately for posterior counting of hatched larvae (eggs on glass and box were always destroyed, after counting - except in the weekends when they were not counted or destroyed). Boxes were changed at least once a week to ensure a highly hygienic condition.

This experiment lasted for 17 days but the treatments were changed on the 12th day (appendix III), in order to detect if pollen did have an effect on oviposition and fertility rate. So, treatment 1 and 2 received the basic diet and aphids for 12 days and the basic diet and pollen for 5; treatment 3 and 4 received the basic diet and pollen for 12 days and the basic diet and aphids for 5 days.

3.4. 500 adults groups

Big plastic cages (14l), open on bottom and top (with glass fibre gauze on the bottom and covered with a transparent plate on top) were used for this experiment. Two small plastic bottles full of honey were attached to the cages (the bottles had a cotton dish adjusted that entered the cage and allowed the ladybirds to feed on the honey). Five hundred, not sexed ladybirds were used, all emerged in the week before or in the week that the experiment started (see appendix IV) and the experiment lasted for 17 days.

A basic diet of *Ephestia* eggs (5 mg/beetle/day) and honey (*ad libitum*) was provided to all treatments three times per week. Treatment 1 consisted of the basic diet plus pollen and frozen aphids once a week; treatment 2 consisted of the basic diet plus frozen adult aphids three times per week whilst treatment 3 was given frozen aphids only once a week, as seen in table 3.4 and appendix VII. However, after 13 days and because the number of laid eggs seemed to be low, the beetles received live instead of frozen aphids (appendix IV). The results section shows how this affected oviposition and fertility rate of the females.

Table 3.4. Experimental setup of the 500 adults groups experiment

Treatment	**Food**	**Feeding scheme**
1	Pollen + aphids 1x week	Honey: *ad libitum* in 2 plastic bottles on top of box, refilled when necessary *Ephestia*: 5 mg/ beetle/ day, provided in several small lids, three times per week (amounts adjusted for the weekend) Pollen: *ad libitum* in 1 big lid; refreshed when necessary (when dry or few pollen present) Aphids: 2 g of frozen ones, divided in 2 small lids, 1x week
2	Aphids 3x week	Basic diet as in treatment 1 Aphids: 2 g of frozen ones, divided in 2 small lids, 3x week
3	Aphids 1x week	Basic diet as in treatment 1 Aphids: 2 g of frozen ones, divided in 2 small lids, 1x week

Initially, three pieces of burlap (10 x 10cm) were placed in the cages but because this proved to be insufficient (the ladybirds were laying more eggs around the cage than in the burlap pieces), that number was increased to six. As in the previous experiments, those pieces were removed every day and the eggs on it and around the cage counted and kept separately for posterior counting of hatched larvae (eggs on the top glass and cage were always destroyed, after counting - except in the weekends when they were not counted or destroyed). From the total number of counted eggs on the burlap pieces, only a random sample of more or less 10 batches of eggs was kept for counting of larvae. The cages were never changed during the whole experimental period but the plastic lids where the food was provided were continuously changed.

4. RESULTS & DISCUSSION

4.1. Individual couples

Even though this experiment lasted for 26 days, all the figures shown are referring to 18 days because the eggs were not collected during the weekends and thus the higher bars in the graphs (every 5 days) represent the number of eggs laid during the weekends. Table 4.1. shows the results for each treatment:

Table 4.1 Results of the individual couples experiment

Ladybirds condition	Continuous rearing (**CR**)			Diapause (**D**)		
Diet	Basic	Pollen	Aphids	Basic	Pollen	Aphids
Treatment number	1	2	3	4	5	6
Total number of eggs/treatment[1]	2112	3338	3921	277	1258	1562
Average number of eggs /treatment [2]	676	714	883	316	313	368
Average number of eggs/couple/day[3]	38	40	49	8	17	20
Fertility rate (%)	54	55	64	39	61	51

[1] sum of the eggs laid by the 5 couples, during 26 days
[2] sum of average number of eggs (thus, eggs per couple), laid per day
[3] sum of average number of eggs/ number of days when eggs were laid

The results show clearly that couples that were reared continuously lay more eggs than those that had been in diapause, independently from the diet. This might have been because D couples started laying eggs later than the CR ones but nevertheless the number of eggs laid in the same period never reached those of the CR couples (as also seen in figure 2). The fertility rate is not consistently higher in the continuous rearing treatments: CR couples fed only the basic diet and fed with aphids did have a higher rate than those in D but not the ones fed with pollen. Figure 4.1 and 4.2 show the daily number of eggs laid during the entire experimental period, for the different treatments and ladybird condition:

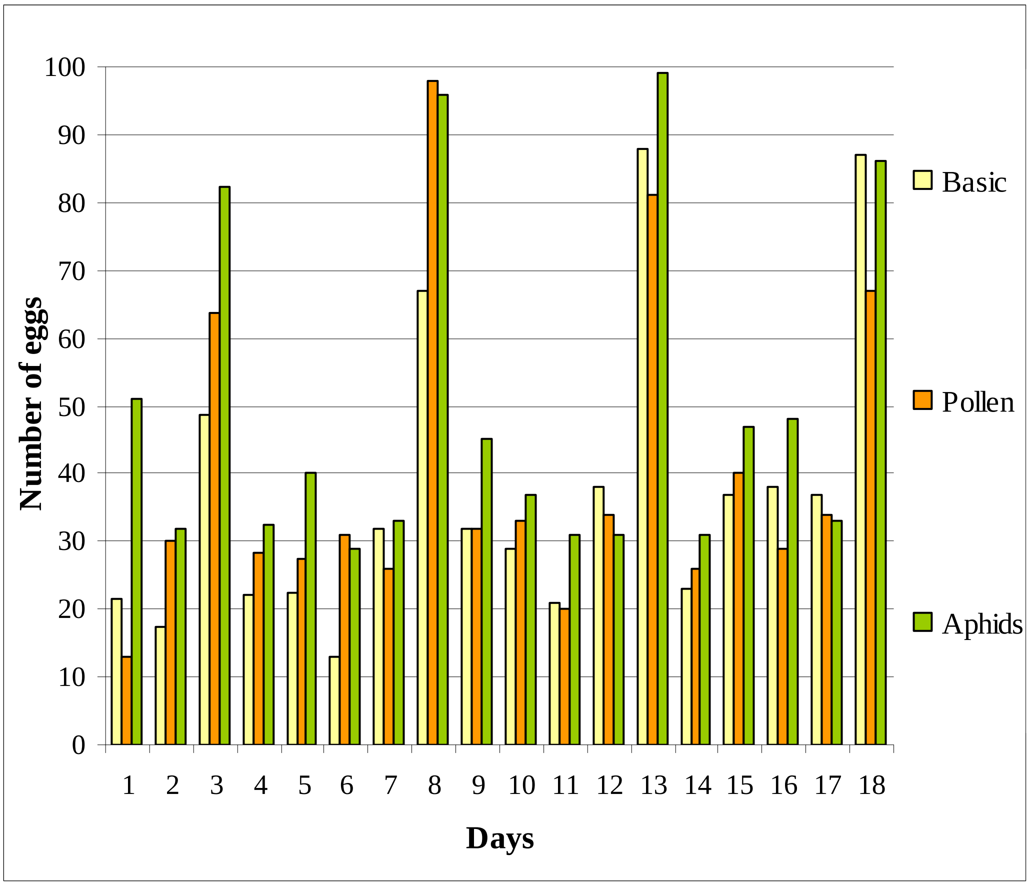

Figure 4.1. Daily average number of eggs per treatment (CR)

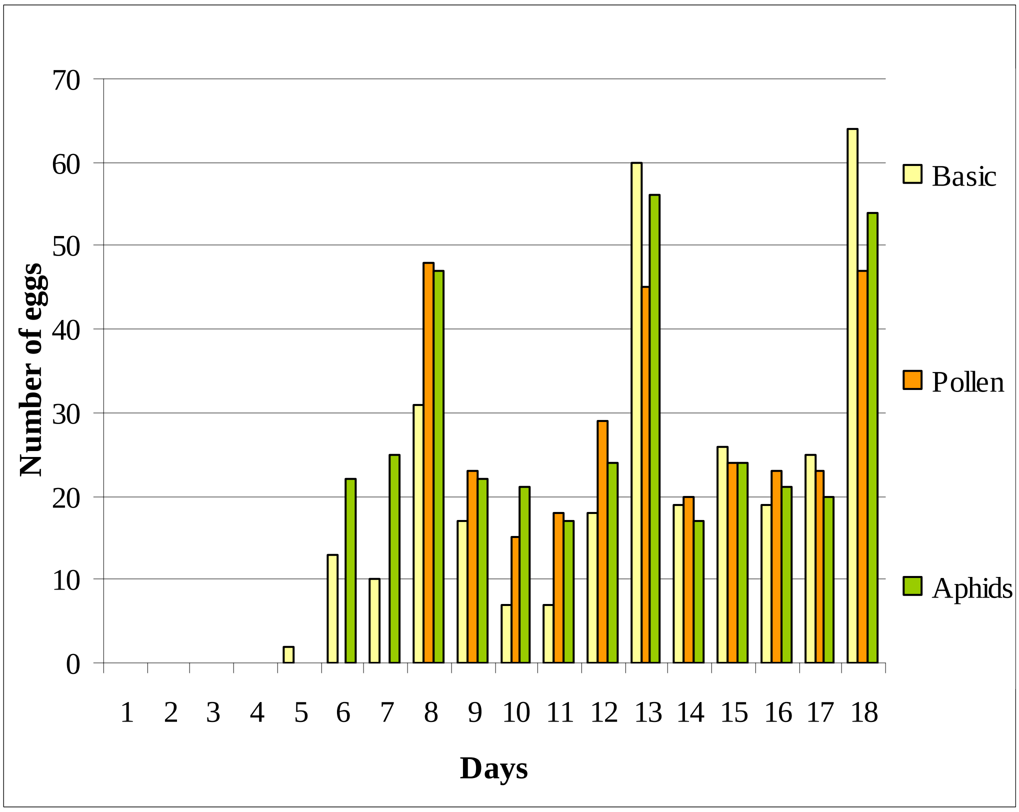

Figure 4.2. Daily average number of eggs per treatment (D)

As for the effect of the different diets within the CR treatments, it is clear that couples fed only the basic diet laid the lowest numbers of eggs and couples fed with aphids the highest (couples fed with pollen laid intermediate numbers). A similar trend is found for the fertility rates. However, the results are strangely different for the diapause treatments: couples fed with pollen laid the lowest number of eggs but with the highest fertility rate, whilst couples fed with aphids laid the highest number of eggs but with an intermediate fertility rate and couples fed the basic diet laid an intermediate number of eggs but with the lowest fertility rate. There is, thus, an obvious effect of the diapause condition in the reproductive performance of the ladybirds. Figure 4.3 shows the total number of eggs laid per couple (or the average number of eggs per treatment) during the entire experimental period, for all treatments and ladybird condition:

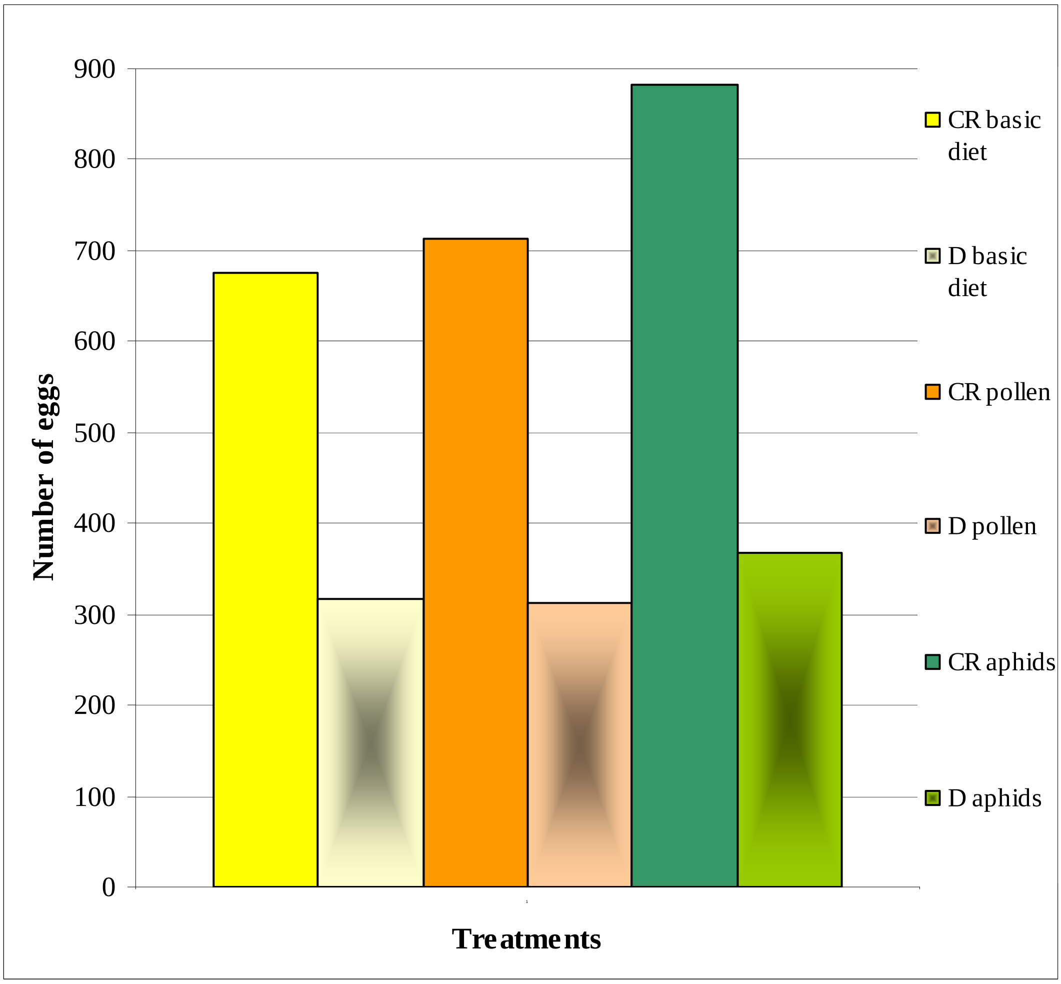

Figure 4.3. Total number of eggs laid per couple, during the entire experimental period, for all treatments and ladybird condition

From these results it is obvious that, at least when kept under optimal feeding and hygienic conditions, the two spot ladybird can lay a considerable number of fertile eggs even when being fed only with a basic diet. Providing aphids does cause a considerable increase in the number of laid eggs and hatched larvae but the increase is not so different from the one caused by the pollen.

The next figure depicts the daily average number of eggs, laid during the 18 days, for all treatments, showing that there is not a linear increase in the number of laid eggs throughout the days. Nevertheless, it is clear that CR treatments laid more eggs daily than the D ones:

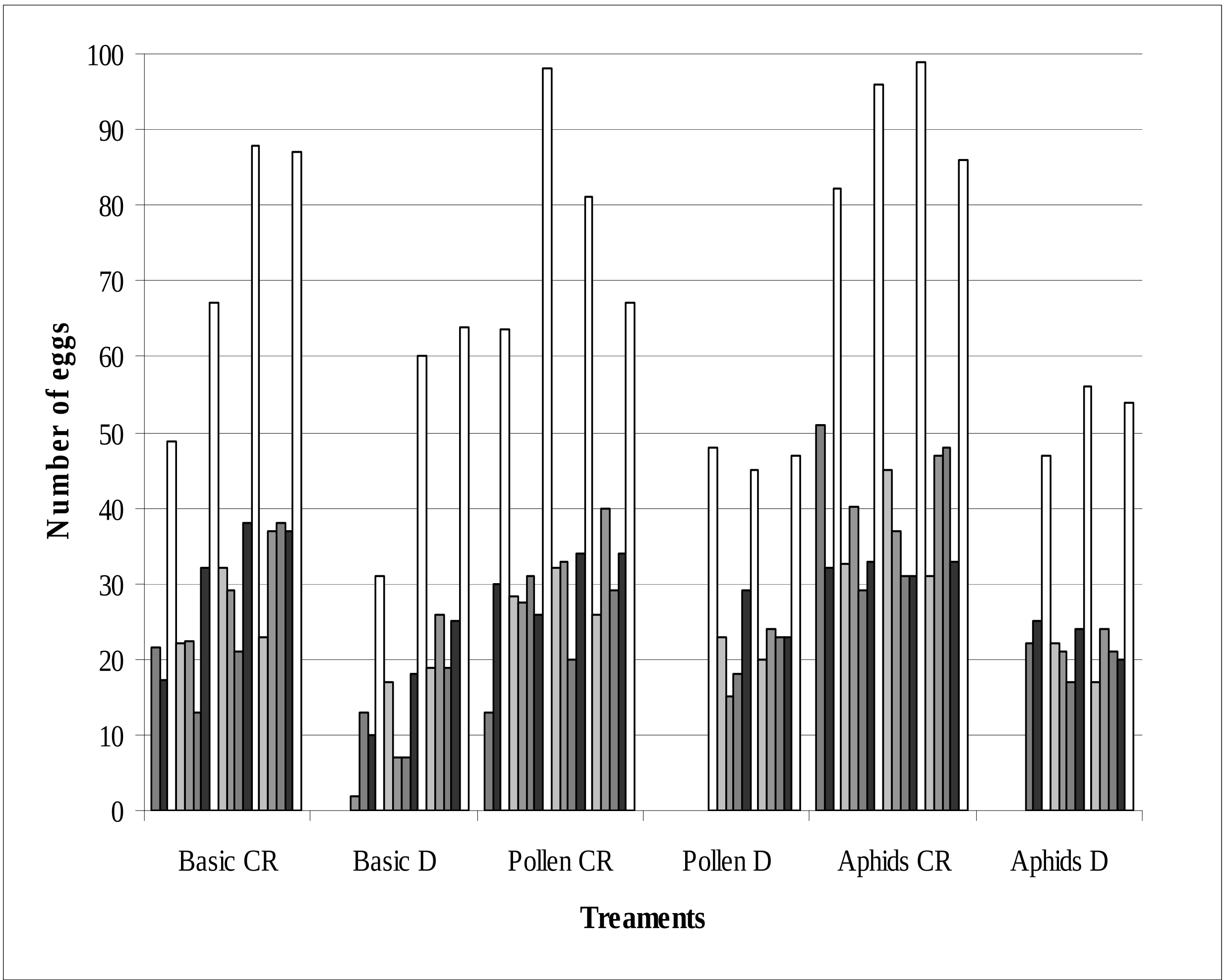

Figure 4.4. Daily average number of eggs, laid during the entire experimental period

The results showed that ladybirds that have spent some time in diapause are definitely not a good asset when the objective of a mass rearing company is to achieve the highest number of eggs possible, in the shortest possible period of time. Even by the end of the experiment, the diapause couples had not laid as many eggs as the continuously reared ones.

Normally, Entocare is not able to produce fertile eggs just by providing the basic diet to the beetles and pollen had never been used. The fact that in this experiment both the basic diet and the pollen gave good results (in the total and daily number of eggs and their fertility) might be explained by the frequency with which the beetles were fed, the good hygienic conditions of the rearing method used and above all, the lack of competition for mates or food.

Because Entocare did not have information on how long can females lay fertile eggs, when fed only the basic food or only aphids, the experiment continued with the best and worst couples until the number of eggs started to decline. The next table (4.2) shows the average number of eggs (average of the best and worse couple) laid by each treatment, during the following 18 days:

Table 4.2 Results of the best and worst couples, after the 26th day

Ladybirds condition	Continuous rearing (**CR**)			Diapause (**D**)		
Diet	Basic	Pollen	Aphids	Basic	Pollen	Aphids
Treatment number	1	2	3	4	5	6
Average number of eggs/treatment	671	617	696	185	409	290
Fertility rate (%)	67	64	62	14	38	33

Within the CR treatments, aphids resulted again in the highest number of eggs but not the highest fertility rate. Pollen treatments laid fewer eggs and with a lower fertility rate than the basic one but there is no explanation for that since all couples had the same age and were treated consistently in the same way.

In contrast, in the D treatments, pollen resulted in the highest amount of eggs and fertility rate, followed by aphids and the basic treatment. Also no logical explanation can be found for these results (except the possibility of “bad quality” of the aphids, even though they were always consumed).

It is clear that after a certain time, the number of laid eggs reduced and that is quite obvious in the D treatments (except for the pollen treatment), as shown in figure 4.6 and table 4.3. The trend is not as clear in the CR treatments (Fig. 4.5). In fact that is a very small decrease in the average number of laid eggs by the basic diet treatments; the difference is bigger in the pollen treatments and the highest in the aphids’ ones, as stressed in the next table (4.3):

Table 4.3. Comparison of average number of eggs laid in the first 26 days (5 couples) and until the experiment was terminated (best and worst couple)

Ladybirds condition	Continuous rearing (**CR**)			Diapause (**D**)		
Diet	Basic	Pollen	Aphids	Basic	Pollen	Aphids
Day 1- 26	676	714	883	316	313	368
Day 26- 44	671	617	696	185	409	290

These results suggest that, when fed only a basic diet, ladybirds will lay an average number of eggs that will stay stable throughout their fertile period. But if the quality of the diet increases, not only the average number of laid eggs increases in the optimum reproductive period (the first month of life), as the difference in laid eggs after a certain time will be bigger.

These two spot ladybirds laid fertile eggs for a total of 44 days, independently from the diet they received, proving that the frequency with which the food is supplied and being kept individually or in groups is extremely important to obtain the most efficient results possible (when comparing the results of this experiment with the ones obtained by feeding the ladybirds with the current scheme Entocare is using). The next two figures show the average number of eggs laid by the best and worst couples during the entire experimental period (from day 1 till 44), showing that the number of laid eggs started to decrease mostly 1 month after oviposition has started:

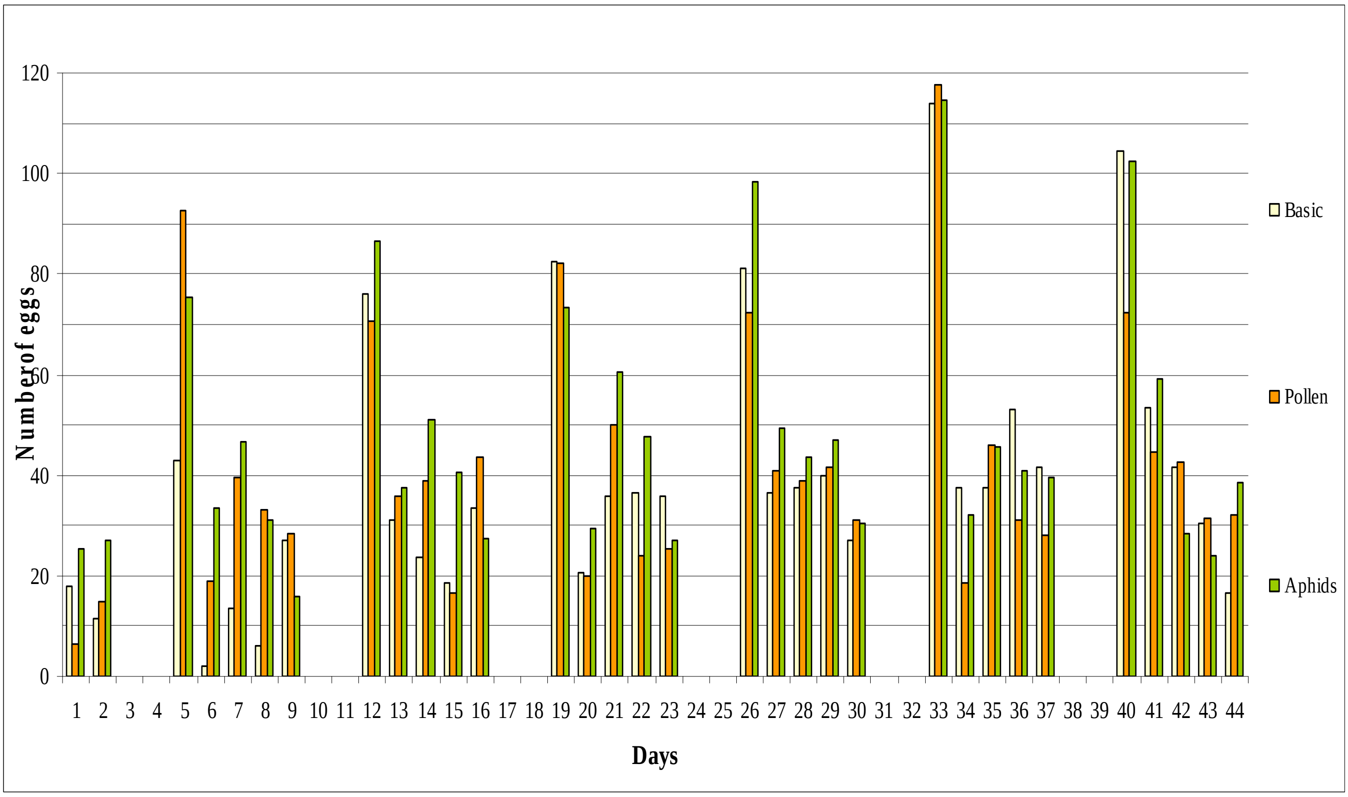

Figure 4.5. Oviposition of CR treatments (average of the 2 couples)

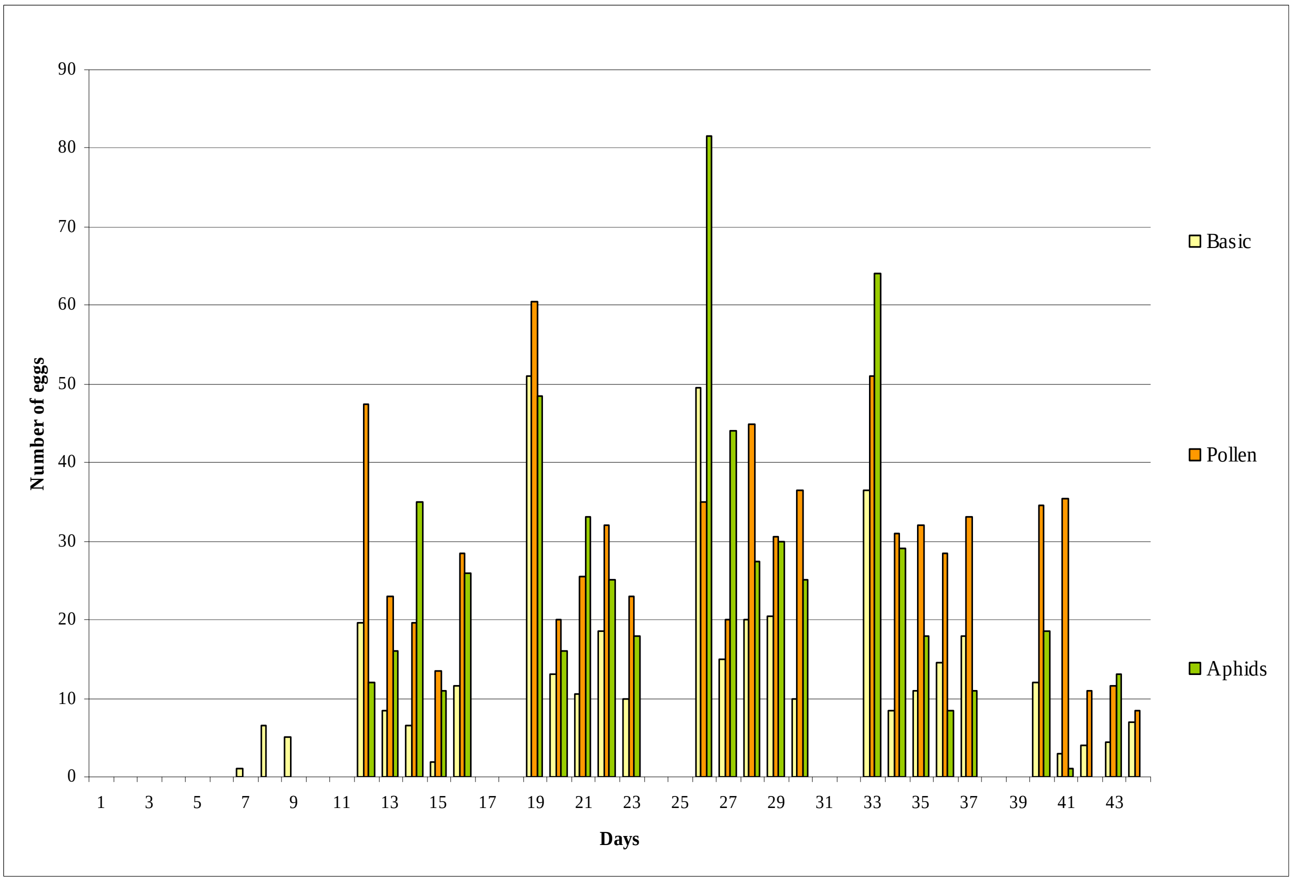

Figure 4.6. Oviposition of D treatments (average of the 2 couples)

4.2. 40 adults groups

Even though this experiment lasted for 17 days, the results will be shown only for 15 of them since in the first 2 days not all the eggs were accounted for, due to practical reasons. The reader should also keep in mind that even though the diet of the groups was switched on day 12, the name of each treatment remains the same in all tables and figures presented (thus, after day 12, groups aphids should be understood as pollen groups and the pollen ones as aphids groups).

The next table shows the several results obtained but special attention should be paid to percentage of missed eggs (laid around the boxes and thus not collectable and unsuitable for packing and sale) and to the number of eggs laid before and after the switch in the diet.

Table 4.4. Results from the 40 adults experiment

Group	**Aphids 1**	**Aphids 2**	**Pollen 1**	**Pollen 2**
Diet	Basic diet + aphids		Basic diet + pollen	
Total number of laid eggs	3954	4112	3711	1936
Total number of eggs on burlap	3047	3168	2976	1821
Total number of missed eggs (%)	23	23	20	6
Fertility rate (%)	66	65	44	53
Total number of **eggs laid before** the diet switch	3325	3094	2445	1175
Total number of **eggs laid after** the diet switch	629	1018	1266	761

The results show that groups fed with aphids laid more eggs than the ones fed with pollen but this difference in not very clear (notice the small difference in the total number of laid eggs between group pollen 1 and aphids 1 and 2). However, there was a difference in the fertility rates: aphids 1 and 2 had a higher fertility rate than the pollen groups but there was not any difference between them. Group pollen 2 laid considerably fewer eggs than group 1 but the fertility rate was higher. These cannot be properly explained, as the number of live adults of group pollen 1 and 2 was not too different at the moment the diet was changed and until the experiment was terminated (see table 4.5).

For both aphid groups, the percentage of missed eggs (laid around the box and not on the burlap pieces) was the same. It is clear that the two spot ladybird does lay many eggs on the box when aphids are provided. This might happen because the aphids were walking freely around the box and as the females forage for them, they laid eggs in their path. On group pollen 2 the percentage of missed eggs was considerably lower, as expected, but there is no clear explanation why group pollen 1 showed a percentage almost as high as the aphid groups.

The next table shows the number of alive couples at 5 different moments of the experimental period and respective number of laid eggs and daily oviposition rates, which can be compared to the results obtained from the individual couples experiment.

Table 4.5. Alive couples and daily oviposition rates at 5 moments of the experiment

Group	Aphids 1			Aphids 2			Pollen 1			Pollen 2		
	Total nr. of laid eggs	Nr. of alive couples	**Daily oviposition/ cp**	Total nr. of laid eggs	Nr. of alive couples	**Daily oviposition/ cp**	Total nr. of laid eggs	Nr. of alive couples	**Daily oviposition/ cp**	Total nr. of laid eggs	Nr. of alive couples	**Daily oviposition/ cp**
Day 1	124	20	**6**	24	20	**1**	62	20	**3**	0	20	**0**
Day 4	904	18	**17**	536	19	**10**	482	20	**8**	133	18	**2**
Day 8	1207	14	**22**	965	20	**12**	882	19	**12**	250	17	**4**
Day 12	1090	10	**29**	1569	18	**22**	1019	18	**14**	679	16	**11**
Day 17	629	3	**50**	1018	13	**16**	1266	14	**18**	761	13	**12**

This table shows how the number of alive couples affected the results, before and after the change in the diet. Very clearly, the number of laid eggs decreased in the aphids groups and increased in the pollen ones, even though the number of alive couples decrease more in the aphids groups than in the pollen ones: it is strange that 3 couples laid more than half of the number of eggs laid by 13 couples (aphids groups) as well as it is strange that 14 couples laid almost twice as many eggs as those laid by 13 (pollen groups). For both, there is no logical explanation but what matters from this table is that, consistently groups fed with aphids laid more eggs than groups fed with pollen. This effect of the switch of the diet in the number of collectable eggs (laid on the burlap pieces) is also clearly shown in the next figure, where it can be seen that before day 12, groups fed with aphids laid more eggs and that after day 12 (when those groups were fed pollen), the groups laid fewer eggs.

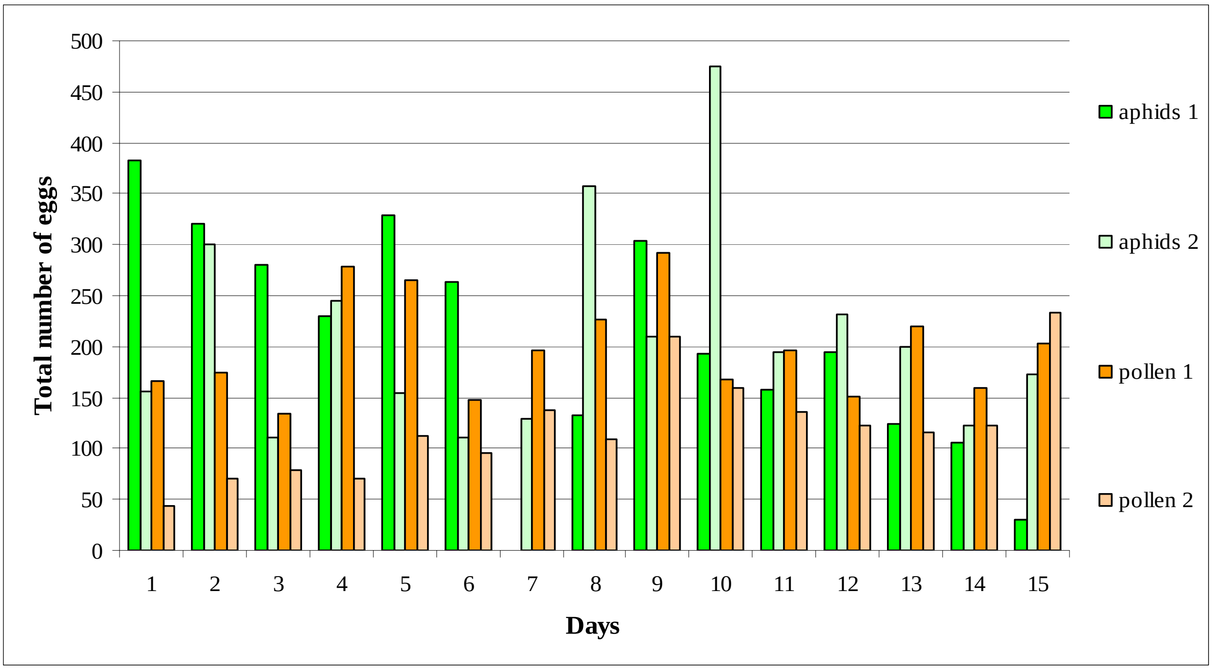

Figure 4.7. Total number of eggs on burlap (all groups): switch in the diet on day 12.

The next figure shows effect of the diet on the total number of laid eggs: during 12 days, aphids groups were fed aphids and pollen groups fed pollen (blue bars) and during 5 days, aphids groups were fed pollen and pollen groups were fed aphids (purple bars).

It is clear that providing aphids, results in an increase in eggs production as it is consistent that those groups always laid more eggs than the ones fed with pollen. Even though the period before and after the switch in the diet is quite different (12 vs. 5 days), the effect is clearly seen right after the day the diet was changed (also seen on day 13 in the previous figure).

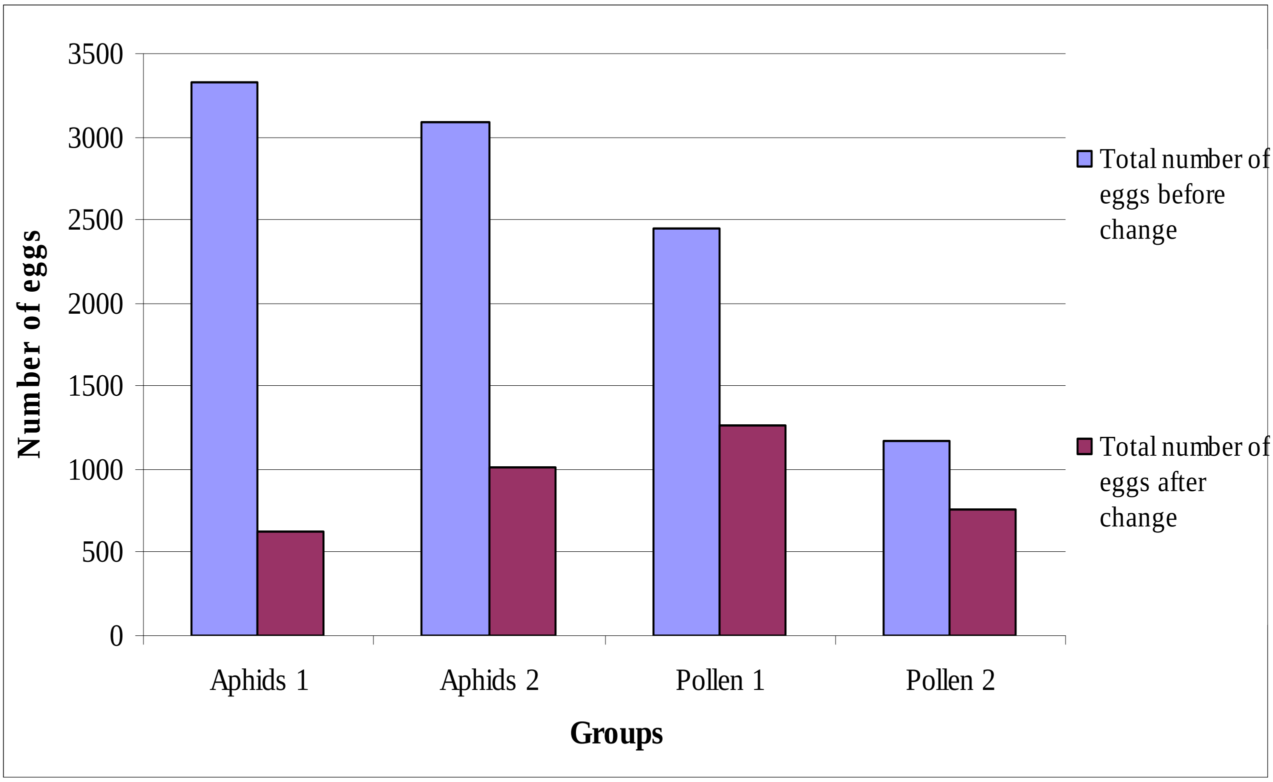

Figure 4.8. Total number of eggs before and after changing the diet (all groups)

4.3. 500 adults groups

The results of this experiment are shown in the next table, where it can be immediately seen that the pollen group laid the highest number of eggs, on the burlap pieces and number of batches around the cage. Strangely, the group fed with aphids three times per week did not result in the biggest number of laid eggs.

Table 4.6. Results of the 500 adults groups experiment

Group	Pollen + aphids 1x	Aphids 3x	Aphids 1x
Total number of eggs on burlap	3389	2575	3139
Total number of eggs kept for fertility rate	1894	1774	1705
Fertility rate (%)	39	38	40
Total number of batches on burlap	403	297	374
Total number of batches on cage	594	248	299
Total number of destroyed batches on burlap (unviable)	278	191	178

Daily oviposition rates (per couples) are not presented in this table (and thus not available for comparison with the individual couples experiment) because their calculations could not be done, due to two reasons: one, the number of dead or missing beetles was not precisely scored during different moments of the experiment (hence, assuming for instance, that 100 eggs laid by 500 beetles on day 1 is the same as 500 eggs laid by 500 beetles on day 17, is a huge mistake). For sure, the number of beetles was reduced throughout the experiment (the number of deads was roughly scored on day 10) and it was not possible to know exactly how many couples laid how many eggs (also because the beetles were not sexed before the experiment started). Second, there was a considerable amount of eggs laid on the cages (in fact, for the pollen group, more batches of eggs were laid around the cage than on the burlap pieces). This exact number of eggs was not possible to count but only the number of batches (by visual observation it was possible to prove that the number of eggs per batch differed a lot). Thus, calculation of oviposition rates based on these numbers is underestimating the number of eggs that the ladybirds really laid.

Figure 4.9 shows the total number of laid eggs on the burlap pieces, for all treatments: ladybirds fed with pollen and aphids once a week laid more eggs than the ones fed with aphids once or three times per week. The group fed with aphids three times a week resulted in the lowest number of laid eggs on the burlap (and number of batches around the cage). From the samples kept apart to check larval hatchability, no big difference was found as the fertility rates of all groups were quite similar to each other.

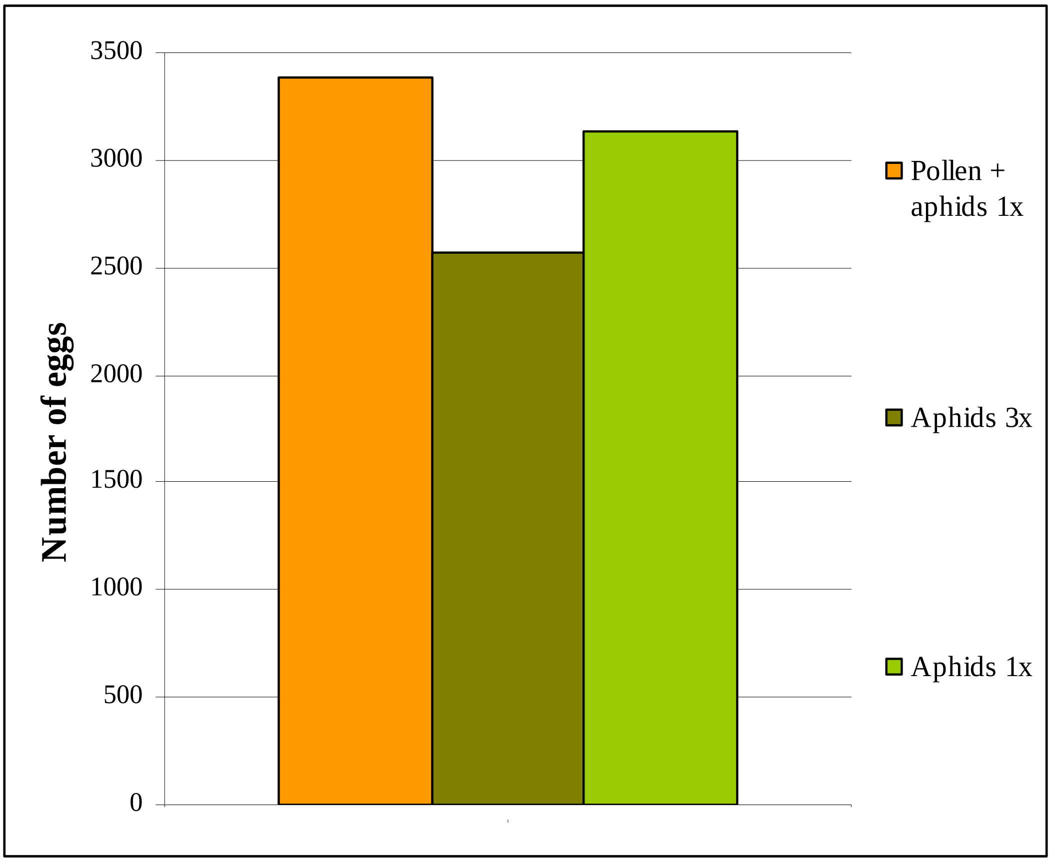

Figure 4.9**.** Total number of laid eggs, all treatments

From table 4.6., it is also understandable that many batches of eggs on the burlap pieces were lost due to destruction by the beetles themselves. The most logical explanation seems to be that they were eaten (by the ladybirds) and destroyed by physical contact of the ladybirds and/or the aphids. If some nutrient deficiency was felt by the beetles, it is normal that they compensated for it by eating the eggs and thus, normal that the pollen groups destroyed more bacthes. Strangely, the groups fed with aphids three times per week destroyed more batches than the one fed only once a week (maybe here, the physical destruction by the aphids seems more likely). It is clear that Entocare has to take this loss into consideration.

It can be discussed that the two spot ladybird does need aphids in her diet to lay as many eggs as possible and for them to be viable but that need might be compensated by providing pollen and less frequently, aphids. The fact that the fertility rates obtained here are lower than the ones from the previous experiments, might be explained by the higher number of beetles kept per cage and not by a deficiency in the diet: groups of 500 adults are under a bigger stress (competition for food and mates) and the diet provided was definetely of a better quality and larger quantity than it is known to be necessary.

The next figure compares the number of batches of eggs laid on the burlap pieces, around the cages and destroyed on the burlap pieces (unviables for packing and sale):

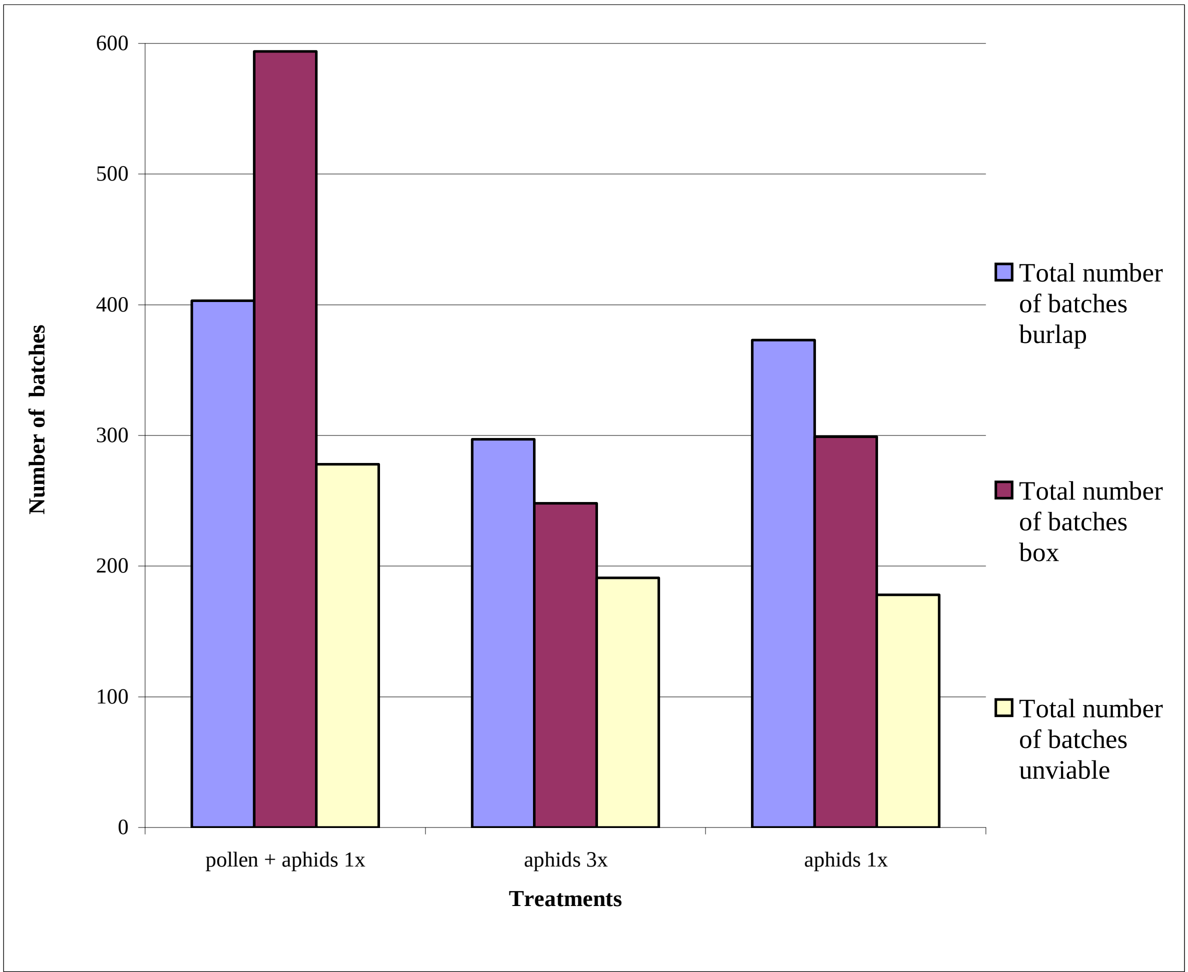

Figure 4.10 Number of batches of eggs on burlap, box and destroyed on burlap

It is clear that the pollen group laid the highest amount of batches on the burlap but also around the cage and these beetles destroyed more batches than the ones in the other two groups. There is no logical reason for the high number of laid eggs around the cage and why that number was so different from the group fed with aphids once a week (the amount of given aphids was the same for both groups). Strangely, the group fed with aphids more frequentely (three times a week), laid the smallest number of batches around the cage but destroyed an intermediate number of batches on the burlap pieces (less than the pollen group and more than the group fed with aphids once a week). If more aphids were given, fewer batches of eggs should have been destroyed than in the two groups.

Figure 4.11 shows the daily oviposition for all groups, during the entire experimental period. It can be seen that the results are highly variable and that the number of laid eggs by each treatment started decreasing after day 10. All dead beetles were removed and counted on this day and thus the numbers presented here, from day 10 till 17 represent eggs laid by some 400 beetles instead of 500 (the number of dead beetles was not very different between groups: 77 for the pollen one, 84 for the group aphids 3x and 116 for aphids 1x).

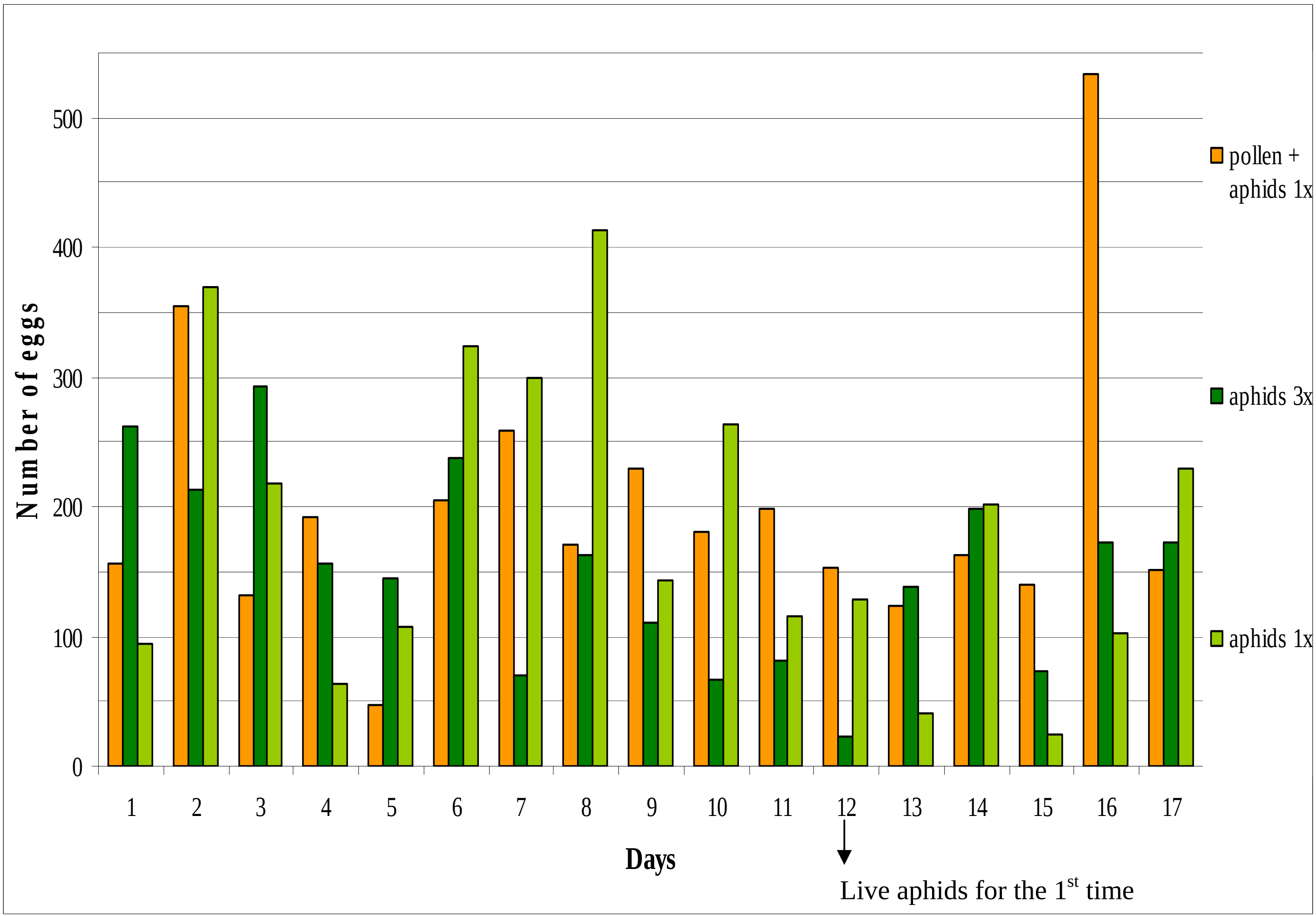

Figure 4.11**.** Daily number of eggs on burlap, throughout the 17 days

From day 12 on, live aphids were given to all the cages (also frozen when live ones were not enough), to try to increase oviposition. That increase in not very clear though, which could maybe be explained by the reduction in the number of live beetles.

It is also clear from this figure, that the results for all treatments were highly variable, throughout the severeal days the experiment lasted and maybe replicating it could help determing the ideal diet for a mass rearing system.

5. CONCLUSIONS AND RECOMMENDATIONS

At first sight, the results of the experiments conducted to find a way of improving the rearing system of the two spot ladybird at Entocare, seemed unexpected but somehow logical.

Females can lay up to 50 eggs per day and oviposit fertile eggs for more than 40 days. There is no doubt that those numbers decrease when the rearing is done in bigger groups and that being kept individually improves their performance as competition for food and sexual mates is not a factor present. Obviously, this is not a solution for a mass rearing company but the **competition for food and sexual mates should be taken more seriously in consideration.**

Using beetles that have been in diapause is definitely not contributing to increase the number of fertile eggs, in the shortest possible period of time. Thus, **diapause ladybirds should not be used in the period of intense production** but only to have an available stock of beetles.

Aphids are indeed necessary for the females to lay as many fertile eggs as possible but that need might be compensated by providing pollen and less frequently, aphids (like once a week). Providing **live aphids does really improve female oviposition but rearing them is only profitable until a certain extent**.

Ladybirds seem to be more productive when living in a very clean environment where food and mates are available. But intense mass rearing cannot afford to invest too much in this and thus clean cages supporting high numbers of beetles must exist. The current cages at Entocare seem to fulfil this (with an open bottom and honey bottles attached on the top) and hygienic conditions are better than when the rearing was done in smaller closed boxes. However, the frequency used to feed the groups with *Ephestia* eggs and aphids can be improved: providing the *Ephestia* eggs more frequently than every 4 days seems to improve female oviposition and fertility (every 2 days) but aphids are also necessary. Some more research should be conducted on the amounts needed but a combination of pollen and live aphids once a week can result in high number of eggs and larvae. Pollen should also be considered as a diet substitute for high numbers of aphids, because when compared to those, pollen is not only cheaper to get (buying vs. rearing aphids, which often does not work well), but also easy to provide and resulting in better hygienic conditions of the cages where the beetles are kept (which results in less hours of work dedicated to cleaning or replacing the cages).

The next table gives a final overview of the research questions the experiments tried to answer and the recommendations suggested to improve the mass rearing system of the two spot ladybird. It seems clear that the number of adults (and their correct sexing), the frequency in which the *Ephestia* eggs are provided, the addition of pollen to the diet and the amount of given aphids are issues Entocare should consider to efficiently increase their production of viable eggs.

Table 5.1. Final conclusions and recommendations

Question	Conclusion	Recommendation
What is the maximum number of eggs a female 2 spot ladybird can lay, when reared under optimal conditions (food, hygienic, environmental, etc)?	Being kept in groups increases considerably competition for food and sexual mates	Adjust rearing to include competition for food and sexual mates (e.g. nr beetles/cage); experiments on effect of sexual competition in a mass rearing should be considered
How is oviposition and fertility rate of a female 2 spot ladybird affected, when kept in diapause between adult emergence and first mating (under optimal conditions)?	Beetles that have been in diapause show lower oviposition and fertility rate	Do not use diapause ladybirds in the period of intense production but only to have an available stock
What is the contribution of pollen and aphids on oviposition and fertility rate of a 2 spot female ladybird?	Providing pollen and live aphids weekly improves female oviposition better than providing aphids more often	Use live aphids as a diet additive once a week but increase the 2g/500 beetles; experiments on the contribution of frozen aphids should be considered

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APPENDICES

Appendix I. Age of the ladybirds used in the individual couples experiment

Ladybird condition	Adult emergence date	Sexing and setting up the couples	Start of the experiment	End of the experiment
Continuous rearing	04 – 07.02.05	09.02.05	09.02.05	07.03.05
Diapause	Last week 12.04	09.02.05		

Appendix II. Individual couples experiment schedule

0	1	**2**	3	4	**5**	6	**7**	8	**9**	10
09 feb	10 feb	11 feb	12 feb	13 feb	14 feb	15 feb	16 feb	17 feb	18 feb	19 fe
Start of the exp	First time eggs were collect									
11	**12**	13	**14**	15	**16**	17	18	**19**	20	**21**
20 feb	21 feb	22 feb	23 feb	24 feb	25 feb	26 feb	27 feb	28 feb	01 mar	02 ma
22	**23**	**24**	**25**	**26**	27	**28**	29	**30**	**31**	**32**
03 mar	04 mar	05 mar	06 mar	07 mar	08 mar	09 mar	10 mar	11 mar	12 mar	13 ma
				End of the exp. Continuing with the best and worst couples						
33	34	**35**	36	**37**	**38**	**39**	**40**	41	**42**	43
14 mar	15 mar	16 mar	17 mar	18 mar	19 mar	20 mar	21 mar	22 mar	23 mar	24 ma
										End o the best and wors coupl exp

Weekends
Feeding days

APPENDIX III. 40 ADULTS GROUPS EXPERIMENT SCHEDULE

1	2	**3**	4	**5**	6	7	**8**	9
07 mar	08 mar	09 mar	10 mar	11 mar	12 mar	13 mar	14 mar	15 mar
Start of the exp. First time eggs were collected.								
10	**11**	**12**	13	14	**15**	16	**17**	
16 mar	17 mar	18 mar	19 mar	20 mar	21 mar	22 mar	23 mar	
		Switch in the treatments					End of the exp	

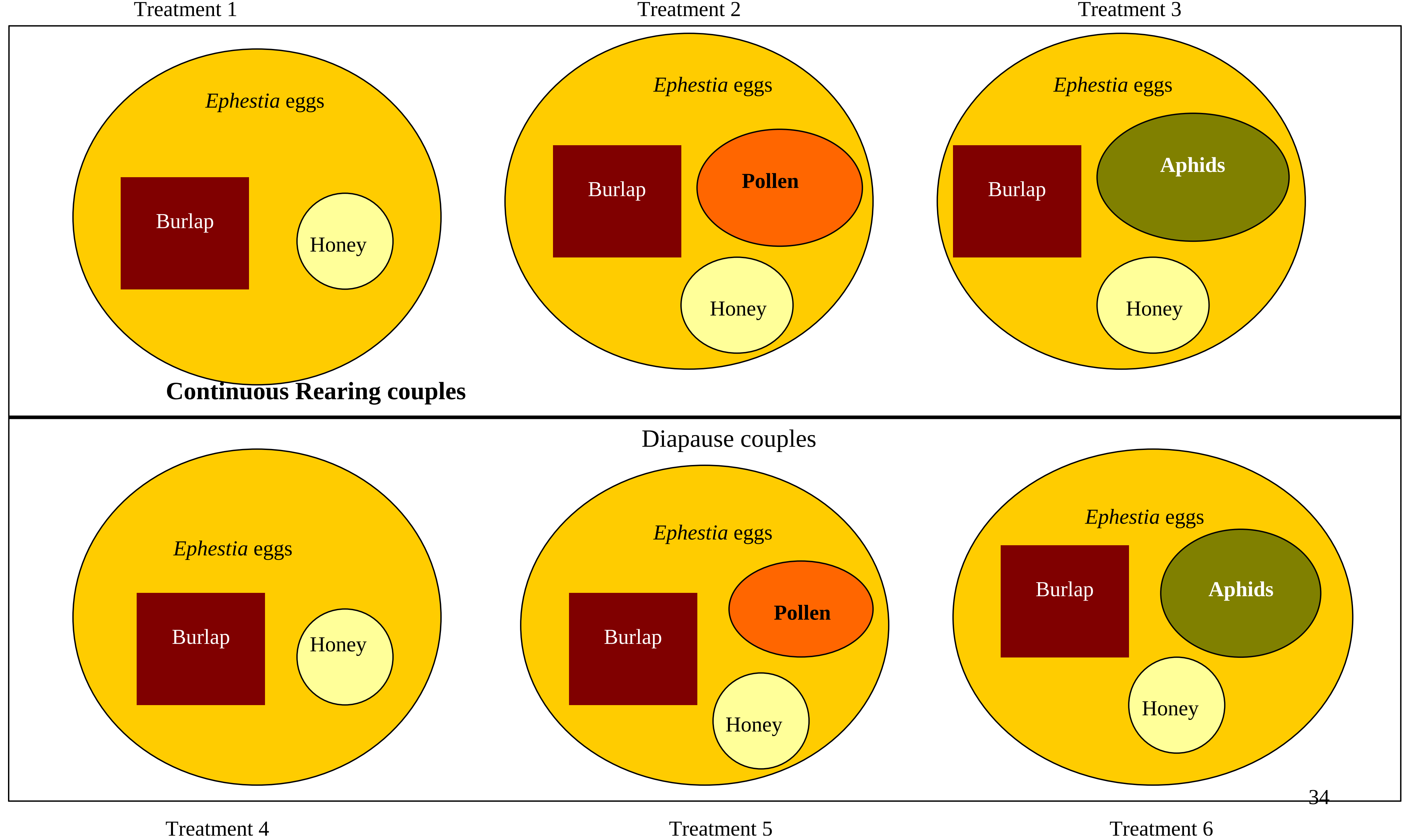
Weekends
Feeding days

APPENDIX IV. 500 ADULTS GROUPS EXPERIMENT SCHEDULE

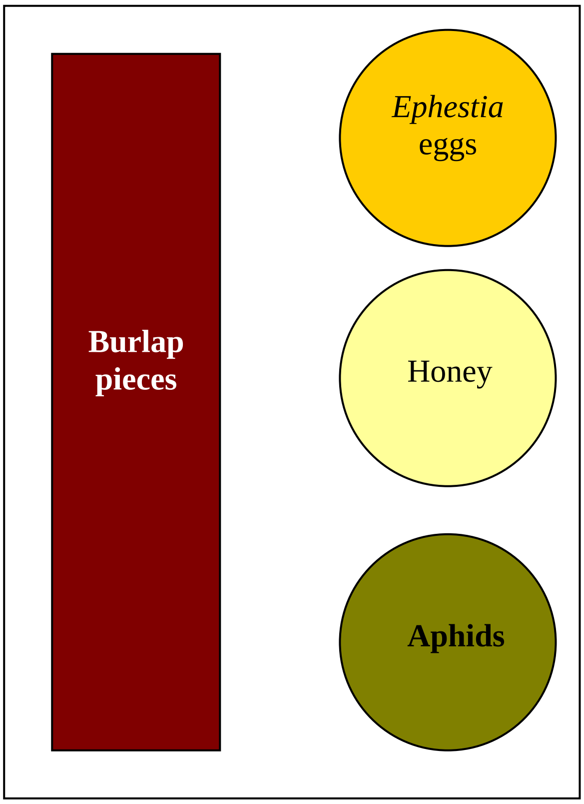
1	2	**3**	4	**5**	6	7	**8**	9
25 mar	26 mar	27 mar	28 mar	29 mar	30 mar	31mar	01 apr	02 apr
Start of the exp. First time eggs were collected								
10	**11**	**12**	13	14	**15**	16	**17**	**18**
03 apr	04 apr	05 apr	06 apr	07 apr	08 apr	09 apr	10 apr	11 apr
			Live aphids were given to all treatments for the first time					End of the exp

Weekends
Feeding days

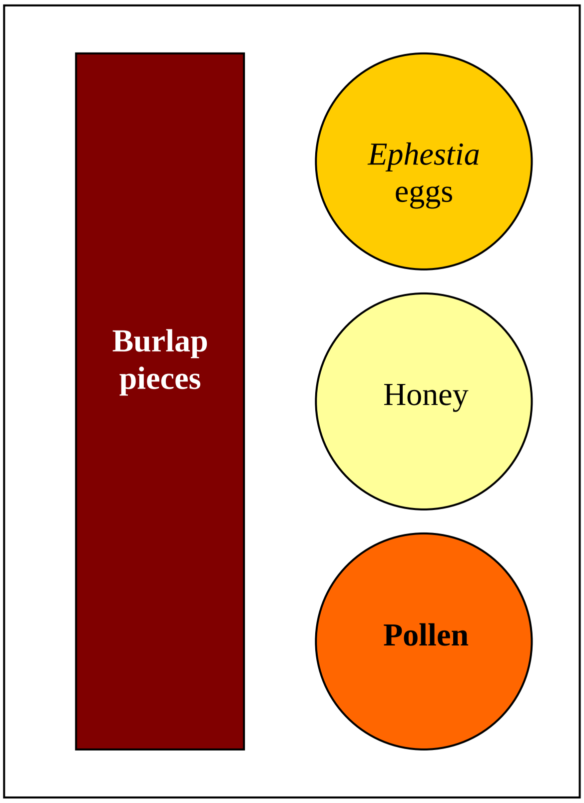
Appendix V. Experimental setup of the individual couples experiment



APPENDIX VI. EXPERIMENTAL SETUP OF THE 40 ADULTS GROUPS EXPERIMENT

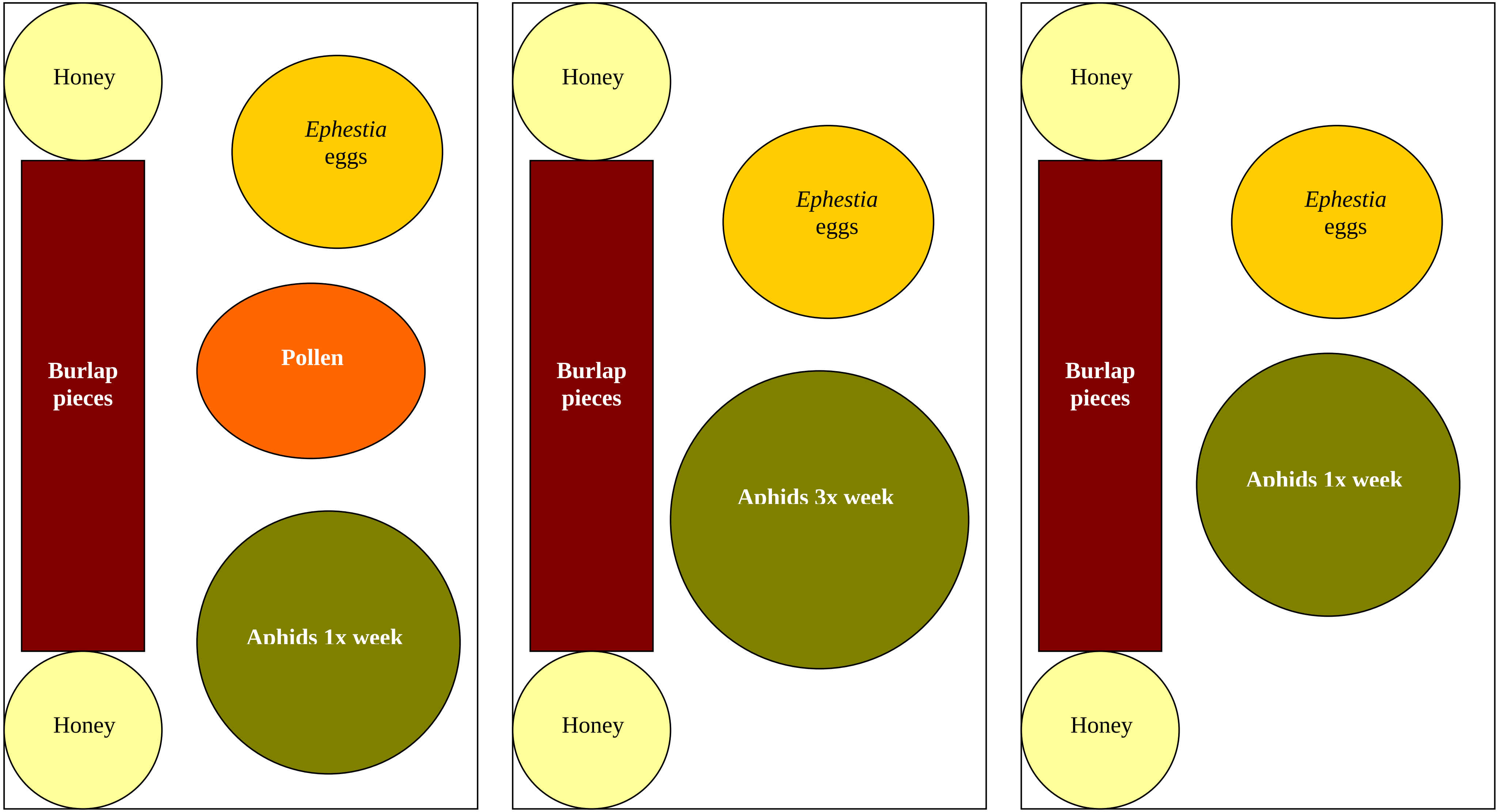


Treatment 1 & 2



Treatment 3 & 4

Appendix VII. Experimental setup of the 500 adults groups experiment



Treatment 1 Treatment 2 Treatment 3