THE USE OF A WINGLESS TWO SPOT LADYBIRD *Adalia bipunctata* (Coleoptera: Coccinellidae) AS A BIOLOGICAL CONTROL AGENT OF APHIDS

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“If we knew what we were doing, it would not be called research, would it?”
Albert Einstein

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PREFACE

Integrated in the scope of my MSc. in Organic Agriculture, I carried out the experiment described in this report as part of my thesis in the Entomology department, at Wageningen University. The theme was part of a broader project, currently being performed in Leiden, on the development of ladybird beetles for improved biocontrol of aphid infestations, under the guidance of Prof. Paul Brakefield.

Fulfilling the objectives of this thesis included periods of: literature study, performance of the field experiment in Leiden (as well as practical work related to the ladybirds, like rearing and learning how to sex them, and aphids identification; all tasks not described in this report), behavioural observations and laboratory essays in Wageningen (some, failed ones, also not described in this report) and posterior elaboration of the report.

I would like to acknowledge all the members involved in this ladybird project, at Leiden university, namely Prof. Brakefield, for his creative ideas and cheerful encouragement, Kees Koops, responsible for the beetles rearing and Niels Wurzer, in charge of all the technical aspects of the polytunnels where the field experiment was performed.

I want to specifically acknowledge: Dr. Peter de Jong for his friendship and kindness, constant guidance and daily support during all the stages of my thesis, without which I could have never finished this report, and for giving me the opportunity of collaborating with the ladybird research group in this new challenging wingless beetle; PhD student Suzanne Lommen, from whom I gained much knowledge in different aspects of the project, from designing the field trial to statistically analyze the results and I hope to have successfully contributed to her own project.

Finally, I would like to say that it was a pleasure (very laborious and sometimes fastidious but still, a pleasure) to work on this topic and to learn so much about ladybirds, aphids and all the aspects that such a research project implies. I sincerely wish the best to the ladybirds researchers in proving the efficacy of the little wingless two spot ladybird.

1. INTRODUCTION

1.1. BIOLOGICAL CONTROL OF APHIDS WITH PREDATORY LADYBIRDS

1.1.1. Ladybirds- an introduction

Ladybirds are amongst the most attractive and popular insects on Earth. Generally, people find them funny and are not afraid (as opposed to reactions to many other insects!) of this red beetle with black spots (colour variation in the 2 spot ladybird will be further explained later in this chapter). For centuries they have been a sign of luck and good fortune, being most of the times associated with religious meanings. In fact, the English name ladybird is a dedication to Our Lady, The Virgin Mary and allegedly comes from the most common British species, the 7 spot ladybird: the red colour is supposed to represent the red cloak with which The Lady Mary is usually seen, in old paintings and sculptures, and the number seven associated with the seven joys and the seven sorrows of The Virgin (Majerus, 1994).

As beetles, ladybirds are part of the Coleoptera order, which is the biggest one in the animal kingdom. Beetles can be characterized as having a large and mobile prothorax and a much reduced mesothorax, large compound eyes, variable antennae, complete metamorphosis and they can be from minute to large insects. Even though insect classification can be difficult and controversial, beetles can be easily differentiated from others due to two special features: they have biting mouth parts and the front wings are modified into hard, thickened wing cases (also called elytra), which cover the membranous hind wings (the true flying organs) (Hodek, 1973 and Majerus, 1994).

Specifically for the 2 spot ladybird *Adalia bipunctata*, the taxonomic classification is as follows:

Class	**Insecta**
Sub class	Pterygota (winged insects)
Infra class	Neoptera
Super order	Endopterygota (or Holometabola- wings develop internally from imaginal discs and there is a marked change- metamorphosis during life history)
Order	**Coleoptera**
Sub order	Polyphaga (hind coxae distinctions)
Infra order	Cucujiformia
Super family	Cucujoidea (tarsal formula and antennae features)
Section	Clavicornia
Family	**Coccinellidae**
Sub family	Coccinellinae
Tribe	Coccinellini
Genus	**Adalia**

Being part of the coccinellidae family literally means “clad in scarlet” (from the Latin translation *coccinatus*) but there are many mimics (other insects that resemble ladybirds) as well as ladybirds (or better, beetles) that wouldn’t be identified as such by a layperson (Majerus, 1994).

Adalia bipunctata is a common colour polymorphic species in Europe, with three main colours in most populations: one *typical* morph which is the famous red beetle with two black spots, one on each elytra and two melanic morphs, the *quadrimaculata*, with four and the *sexpustulata*, with six red spots on a black background. Determination of this colour variation is controlled by a single gene (Lus, 1932). Besides these most obvious morphs (because well shaped spots are readily noticeable), this ladybird presents an amazing colour pattern variation of more than a hundred different colour patterns that range from being all red to all black (Majerus, 1994).

Ladybirds have usually a one year life cycle. They usually aggregate in big groups to hibernate in shelters (usually high places) during the winter and become active in spring when climatic conditions are favorable. They will then forage for food and after that start looking for a mate (Hodek, 1973). Searching behaviour for a mate is not well explained and it seems that males just seem to find females by bumping into them. Most species are highly promiscuous and in the two spot ladybird, repeated matings are necessary to maintain a high level of egg fertility (Majerus, 1994). The two spot males are also known for not properly distinguishing between compatible partners and there are reported cases of males trying to mate with other males, females from other species and even dead individuals (Majerus, 1994). Mating can last from one to eight hours and females will lay twenty to fifty yellowish eggs a day, in batches that become grey before larvae hatch (Hodek, 1973 and Majerus, 1994).

The duration of larval instars depends greatly on weather conditions and food abundance but typically, lasts three to four weeks. When a fully grown larva stops to feed, it is ready to pupate. One to two days before pupating, it will become immobile and attaches itself to a substrate. Depending on temperature, an adult will emerge from the pupa one week or so after pupation (Hodek, 1973). Adult stages of the two spot ladybird will become reproductively mature approximately one week after emergence (Majerus, 1994 and Blackman, 1974).

1.1.2. Ladybirds as biological control agents of aphids

Ladybirds are highly polyphagous and eat plant pests, from aphids to coccids and mites. Nevertheless, they are not too picky with their food and when their favourite (aphids) is not present, they will feed on honeydew, pollen, nectar and even mildew (Majerus, 1994).

In the wild, they are the most efficient natural enemies of aphids and have been used for a long time as biological control agents in greenhouse production ((Hämäläinen, 1977). Several studies have been conducted throughout the years regarding specificity and voracity of the two spot ladybird and suitability of different aphid species and their consequences for reproductive performance (Özder & Sağlam, 2003, Kalushkov, 1998, Mills, 1982, Hämäläinen, 1975 and Blackman, 1965). This has allowed further investigation of the efficiency of the two spot ladybird as biological control agent and proper management programmes have been designed. However, there has always been a problem regarding their dispersal by flight and usually, control of aphids in greenhouse crops involves costly introductions of the beetles (Hämäläinen, 1977), making the use of parasitoids a more successful technique to use.

So, it seems then that the two spot ladybird is unlikely to be an efficient predator of aphids due to inherent disadvantages (Mills, 1982). They should leave an aphid aggregate as soon as a certain number of eggs are laid and/ or larvae are present, even though aphids still increase in abundance (Kinglmann & Dixon, 1993). So, the specific particularities in the relationship aphid-ladybird lifecycle and the limited voracity or inefficient searching behaviour of the ladybird (Minks & Harrewijn, 1988) make the common wild type *Adalia bipunctata* a not so successful biological control agent.

1.2. BACKGROUND STORY ON THE WINGLESS LADYBIRD

A wingless male specimen of *Adalia bipunctata* was found in a wild population, under lime trees in 1990, in a locality near Utrecht in The Netherlands. Contrary to wingless individuals found before (Majerus and Kearns had already reported, in 1989, the existence of such individuals but they could not turn over when they fell on their backs, so eventually died), some of these ones could turn over by sudden flicking of the abdomen or by curling themselves sideways and then rolling over, which is not known in the winged forms of the two spot ladybird (Marples *et al*., 1993).

Through breeding experiments, Marples *et al.* (1993) were able to show that the wingless trait was inherited as a recessive allele which displays different levels of expression and they concluded that wingless individuals are homozygous for a recessive gene which prevents normal development of the elytra and flight wings. Unfortunately, none of the F4 bred ladybirds produced viable offspring and the wingless stock was eventually terminated. Majerus (1994) mentioned in his Ladybird book the existence of these two spot individuals where the elytra and wings could be reduced to miniature stumps but no more special attention was given to these odd ladybirds.

Luckily, in 2000 Dr. Ueno repeated the discovery of a wingless individual at the same locality in The Netherlands and bred a large stock under controlled conditions (pea aphids reared on bean plants were given as diet). Dr. Ueno and his colleagues confirmed the recessive mode of inheritance of the trait and also found that there is a highly variable expression of the wingless homozygous condition. Maintaining a healthy stock was no problem even though it appears that the wingless phenotype has a low reproductive potential (in laboratory conditions, wild type and wingless adults eat comparable numbers of aphids with equal voracity). Ueno *et al*. (2004) also found that wingless individuals show a longer developmental period, lower egg production and shorter lifespan than the wild type with wings and they suggested there might be a consistent pattern of correlation between the degree of winglessness and life history traits (most extreme wingless individuals showed the lowest fitness while those with more developed wing buds tended to have the highest fitness).

The contribution of the research in the genetics behind the wingless condition and its consequences on reproductive and predacious performances, brought up an interesting question regarding the use of these wingless ladybirds as more efficient biological control agents of aphids in greenhouses, as they might remain longer on the plants (since they have no wings to fly off), whilst winged individuals will disperse by flight once aphids decline below a critical threshold density (Hämäläinen, 1977).

Wing polymorphisms occur widely in the insect world (Roff, 1984) and winglessness is a common phenomenon, with loss of flight wings occurring in nearly all pterygote insect orders (Edmunds, 1992). Wing evolution has allowed insects to escape their predators, exploit resources and disperse into new niches and in the evolutionary history of insects, thousands of transitions have occurred from a winged form to a wingless one (Whiting et al., 2003). Like in the two spot ladybird, many insect species show wing dimorphism, with one morph having fully developed wings and the other having reduced wings and thus incapable of flight (Roff & Fairbairn, 1991).

Many authors have discussed the possibility of a trade off between flight ability and the cost of producing and maintaining wings and flight muscles and in most wingless forms, a higher fecundity and earlier age of first reproduction has been observed (Ueno *et al.*, 2004). However, that has not yet been reported for the wingless two spot ladybird.

Specifically in the Coccinellidae family, there are studies about the use of flightless *Harmonia axyridis* (the multicoloured Asian ladybird) and how to improve its efficiency as a biological control agent of aphids and wingless individuals were found more effective (Kuroda & Miura, 2003) probably because adult stages have a higher predation capacity and flightless individuals remain longer on the plants. Ferran *et at*. (1998) produced artificial non-flying mutant individuals and their study showed that the wingless beetles remain on the plants in higher numbers and lay eggs over a longer period of time but their progeny is less numerous. These individuals seem to show a loss of fitness (higher mortality and lower fecundity and fertility) that prevents their use in biological control of aphids but nevertheless, Ferran *et al.* (1998) did indicate that the inability to fly seems to increase the number of adults on the plants. Releasing flightless adults of this ladybird in aphid infested greenhouse crops seems thus to have the potential for a more continuous control of aphids (Tourniaire *et al*., 2000) due to the behavioural and biological features of these wingless beetles (Tourniaire *et al*., 1999).

1.3. Scientific aims of the project

A broader project has been planned, at Leiden University to study different aspects of the development of ladybird beetles for improved biological control of aphid infestations, including improvement and development of the existing genetic stocks of the wingless morph, production of new and more effective stocks by artificial selection experiments and finally research on the genetic and developmental mechanisms underlying the wingless condition.

This ambitious proposal, designed to increase the effectiveness of ladybirds for biocontrol of aphids, implied the field experiment described in this report with the specific research aim of quantifying that effectiveness through measurements on the efficiency of the wingless ladybird as an aphid predator (by monitoring both aphid and ladybird populations, in field conditions and trying to understand those results with behavioural observations in laboratory conditions).

2. MATERIALS & METHODS

2.1. Field Experiment

The setup of this field experiment was carefully planned in advance according to the research objectives. However, because this was a first trial in a much broader set of trials, many decisions regarding its experimental design were made based on daily observations of how the plants, aphids and ladybirds were developing. Hence, the detailed explanations in the following section are presented so the reader can better understand why this field experiment was done in such a way.

2.1.1. Experimental setup

The experiment described in this report was conducted at the Einsteinweg garden, in Leiden, The Netherlands between June and September 2004.

A semi field condition was created with the construction of a plastic polytunnel of 20 x 6 m that contained 768 strawberry plants, *Fragaria* species, variety El santa. The polytunnel was previously divided into four compartments, by an aphid-proof net and two wooden rows to support the trays containing the plants were built, per compartment.

The ladybirds used in this experiment were reared in laboratory, at Leiden University, section of Evolutionary Biology (appendix I). Winged specimens were heterozygotic for the wingless trait, obtained by crossing wild and wingless ladybirds and their offspring reared in laboratory and wingless specimens of *Adalia bipunctata* were descendents from a stock being reared in laboratory conditions for a long time. It included a total of 768 ladybirds, divided between the winged and wingless condition, in a design resumed in table 2.1 and shown in the map in appendix II:

Table 2.1. Experimental design of field experiment		Total
Compartments	2 wingless & 2 winged	4
Ladybirds	1 ladybird released per plant	768
Plants	6 plants per tray (50 x 50 cm each)	768
Trays	16 trays per row (8 m long each)	128
Rows	2 rows per compartment	8

The young strawberry plants used had been frozen for over the winter and spring and were grown in a biological way, without any chemical control. Water and nutrients were supplied in small amounts, several times per day through a dripping system. All pest management operations (appendix III) and pollination of the plants to guarantee fruit production was done with the use of biological agents from Koppert BV.

Because the occurrence of aphids at the cooperating growers at the time of the experiment was quite reduced and hence no sufficient numbers were available, the decision was made of letting spontaneous outbreaks of this pest occur inside the polytunnel compartments. The result was that a few weeks after the setting up of the experiment, the plants were slightly attacked by aphids and their numbers were allowed to increase until the ladybirds were released (appendix IV). After collection and identification of the different species present it was concluded that the plants were mainly infested with the known glasshouse- potato aphid *Aulacorthum solani*. *Macrosiphum euphorbiae*, the potato aphid was also identified but in much lower numbers.

2.1.2. Measurements & observations

Monitoring of the aphid population started right after the plants were considerably infested. Classes of aphids were used to estimate their numbers in different plant structures (Table 2.2). Table 2.3 shows how the plants were divided into reproductive and vegetative parts so that later only the most important ones would be used for convenient discussion (most important ones being defined as those plant structures where higher classes of aphids were scored).

Table 2.2 Aphid classes

Aphid classes	
0	0 aphids
1	1 aphid
2	2 to 5 aphids
3	6 to 10 aphids
4	11 to 20 aphids
5	21 to 50 aphids
6	51 to 100 aphids
7	More than 100 aphids

Table 2.3. Plant structures

Plant structures			
Vegetative parts		Reproductive parts	
1	Leaf	1	Flower
2	Stem leaf	2	Stem flower
3	Young leaf	3	Fruit
4	Stem young leaf	4	Stem fruit
5	Bud	5	Stolon
6	Stem bud	6	Stem stolon

The division of the plants into the different parts was also done in order to facilitate estimation of the aphid numbers as the plants were developing quite fast and changing their shape from one measurement day to another. Appendix V shows how a strawberry plant develops into these different structures. The emergence of young leaves on the plants was continuous and a consequence of bud development: as time passed, buds grew into young leaves and new buds emerged at the crown of the plants. However, these young leaves were only scored for aphid density after measurement 6, when there was a clear distinction between new buds at the crown of the plants, young leaves (smaller in size and height and lighter in colour) and older ones.

The scoring and monitoring of the aphid population was done two times a week during a total period of 6 weeks (appendix IV). Until the introduction of the ladybirds, the measurements were taken always from the same plant and one only, in every tray (so, a total of 128 plants were scored: 16 plants x 8 rows) but after the release of the beetles the measurements were taken only from a plant in every second tray (but always in the same plant as before), meaning that a total of 64 plants were scored (8 plants x 8 rows). This was done only due to the huge amount of time necessary to take the measurements.

From the moment that the ladybirds were introduced, the measurements were taken twice a week to a total of 6 measurements, as stressed in appendix IV.

Release of the ladybirds in the polytunnel was preceded by its division with the nets into four compartments, two wingless and two winged ones. Placement of each wing state into the different compartments was done considering that climatic conditions could vary in each part of the polytunnel: front, back, left or right side (Appendix II). All the ladybirds used were approximately the same age and a strong effort was made to place equal numbers of both sexes and morphs (*typica* and melanic forms), in every row, as shown in the following scheme:

> 1 ladybird per plant → 6 ladybirds per tray (3 melanics + 3 *typicas*) x 16 trays → 96 ladybirds per row: 48 *typica* (24 ♀ + 24 ♂) and 48 melanic (24 ♀ + 24 ♂) x 2 rows → 192 ladybirds per compartment x 2 compartments → 384 wingless + 384 winged ladybirds

Wingless ladybirds were individually placed with a tweezer on top of higher leaves, one per plant, with the different morphs equally distributed and the sexes randomly distributed, over each row.

The same scheme was used to place the winged ladybirds, which were forced to fall on the plants, in the middle of the trays but generally they just flew away from the petridish used to transport them from the laboratory to the polytunnel.

Together with the monitoring of the aphid population in each plant, the presence of ladybirds on it was checked as well as position in the plant (in which plant structure the ladybird(s) was). When reproduction took place, the number and position of egg batches and later, the number of larvae were scored. Moreover, migration of the ladybirds within and across compartments was also monitored with their position scored as walls, roof and floor of the compartment. Dead ladybirds were always searched for and their position noted.

Occasionally, observations were done on the presence of other aphid predators and parasitized aphids.

2.1.3. Statistical analysis

The data were analysed by SPSS 11.0 for Windows, using repeated measures ANOVA to test differences between aphid consumption in wingless and winged compartments. Chi-square tests were used to detect differences between the number of ladybirds in wingless and winged compartments and between number of ladybirds on plants or off the plants.

2.2. BEHAVIOURAL EXPERIMENT

2.1.1. Experimental setup

After a preliminary analysis of the field experiment results, behavioural observations inside a cage (appendix VI) were done in laboratory conditions, at Wageningen University to check if indeed wingless ladybirds would remain on the plants and winged ones would rapidly fly away from it to the surrounding cage.

In a temperature room, one strawberry plant used previously in the polytunnel experiment was placed in the center of a wooden and glass cage of 95 cm x 100 cm x 112cm (see appendix VI). The plant had been previously cleaned with a CO_2 flow in order to ensure no parasites or other insects were present (see appendix VII).

Adult *Aulacorthum solani* aphids (reared on plants at room temperature, as seen in appendix VIII, but descendents from the polytunnel population) were released in this plant before the observations were done to guarantee that large numbers were being provided to the ladybirds (about 20 to 30 adult aphids for each set of ladybirds) and thus food stress was non-existent and not influencing the natural behaviour of the beetles. Young aphids appeared every now and then indicating that some reproduction took place among them. Even so, adults were introduced almost daily to assure that the ladybirds were properly satiated.

An attempt was made to replicate the field conditions in terms of ladybird numbers and aphid densities: one ladybird per plant, considerably infested with aphids. However, the setup of these observations had to be adjusted due to lack of time and materials to repeat those conditions. Nevertheless, it can be safely assumed that these laboratory conditions were resembling the field conditions as well as possible, allowing to compare field with laboratory results: winged ladybirds remain on the plants for short (or none) periods of time, as opposed to the wingless ones.

Thus, these behavioural observations were finally designed using a higher number of beetles: each observed set included four ladybirds (two of each wing state), placed in one plant. After a pilot experiment, where one couple of ladybirds was observed, the decision was made of using only individuals of the same sex, in each set because the ladybirds were virgins and it would be normal that if males and females were together for the first time, mating would be the behaviour mostly observed (not fulfilling the objective of these observations).

Ten replicates of each set were done, each set consisting of two wingless and two winged ladybirds. All the ladybirds came from the stock reared in the Leiden laboratory (appendix I), with eclosion date from 29.09.04 ($\pm$ 5 days). Table 2.4 explains the sets and difference in age of each of the ladybirds used:

Table 2.4. Sets, sex and age of ladybirds used in the behaviour experiment

Set nr.	Number of ladybirds	Wing state	Sex	Morph	Date	Age of the ladybirds (days)
1	1	wingless	♀	*typica*	04.11.04	36
	2			*typica*		
	3	winged		melanic		
	4			melanic		
2	1	wingless	♂	melanic	07.11.04	39
	2			*typica*		
	3	winged		melanic		
	4			*typica*		
3	1	wingless	♀	*typica*	08.11.04	40
	2			*typica*		
	3	winged		*typica*		
	4			*typica*		
4	1	wingless	♂	*typica*	09.11.04	41
	2			melanic		
	3	winged		*typica*		
	4			melanic		
5	1	wingless	♀	*typica*	10.11.04	42
	2			*typica*		
	3	winged		*typica*		
	4			*typica*		
6	1	wingless	♂	*typica*	11.11.04	43
	2			*typica*		
	3	winged		*typica*		
	4			melanic		
7	1	wingless	♀	*typica*	15.11.04	47
	2			*typica*		
	3	winged		melanic		
	4			*typica*		
8	1	wingless	♀	*typica*	16.11.04	48
	2			*typica*		
	3	winged		*typica*		
	4			melanic		
9	1	wingless	♂	*typica*	24.11.04	56
	2			melanic		
	3	winged		*typica*		
	4			*typica*		
10	1	wingless	♂	*typica*	25.11.04	57
	2			*typica*		
	3	winged		*typica*		
	4			*typica*		

2.1.2. Measurements & observations

Each set of ladybirds was placed on the plant in the morning, after inspection of the number of aphids present (added, when necessary), by picking them up with a tweezer and letting them go to a leaf surface. The two wingless ones were always placed on the same leaf and the two winged ones also on the same leaf but on a different one than the wingless (both leaves were older and higher ones, not close to the crown of the plant where leaf buds were present). All ladybirds were then situated approximately at the same distance from the aphids' foci, mainly the young leaf buds.

After introduction of the ladybirds on the plant, an immediate observation was done for half an hour, continuously. Another half an hour of continuous observation was done 3 hours after the placement of the ladybirds on the plant. Their location in the cage was scored every hour, between the first and the second continuous observation and every hour after that until the end of the day, when the ladybirds were removed and frozen. This resulted in a total of 8 scores (appendix IX) .The lights of the room were kept on during the entire period the beetles spent inside the cage.

Location of the beetles on the plant and/or around the cage was initially divided in several categories (Table 2.5) but due to lack of time to analyze all the data, only position of ladybird on the plant or on the cage were considered, without any other distinctions for further analysis and discussion.

Table 2.5. Location of ladybirds on plant and around the cage

Plant [1]	**Cage** [2]
L up- leaf upper side	**F-** wooden frame
L un- leaf under side	**Fl**- floor of cage
L ed- leaf edge	**G**- glass (**T**op, sides: **R**ight or **L**eft and **F**ront and **B**ack)
ST- stem	**Pt**- pot
Inter- intersection of petioles from the same stem	**Sl**- soil
Cr- crown	**D**- pot dish

[1] For specific location on the plant, see appendix V
[2] For specific location on the cage, see appendix VI

Several different behaviours were also scored (table 2.6) but differences between them not analyzed statistically and thus not mentioned in the results chapter.

Table 2.6. Behavioural scores

1. Walking	**W**
2. Standing still	**S**
3. Preening	**P**
4. Moving abdomen	**MA**
5. Mating: copulating or just courtship (attempt mating)	**Mt** **Cp** **Ct**
6. Laying eggs	**LE**
7. Eating aphids	**EA**

2.1.3. Statistical analysis

The scores obtained were analysed by means of SPSS 11.0 for Windows, using Chi-square tests, to detect differences between ladybirds' position (number of wingless and winged ladybirds, on the plant and on the cage). Data from each score was analysed separately (for each score, the results from each of the 10 sets were pooled and tested statistically for differences between scores).

3. RESULTS

3.1. FIELD EXPERIMENT

The results of this experiment are shown divided in time, before and after the introduction of ladybirds in the plants. However, several plant structures were scored according to the aphid density but not all of them were used in the statistical analysis thus the next section explains which and why certain structures were kept out of the analysis and subsequent discussion of results.

3.1.1. Plant structures

From the twelve plant structures described in section 2.1. (see appendix V), only four were chosen as the most important ones for further discussion and to show how density of aphids developed in time: leaves, buds, flowers and stems of flowers. The remaining plant structures were excluded because of either too low scores on it or absence of the structure in certain measurements, as illustrated in figure 3.1:

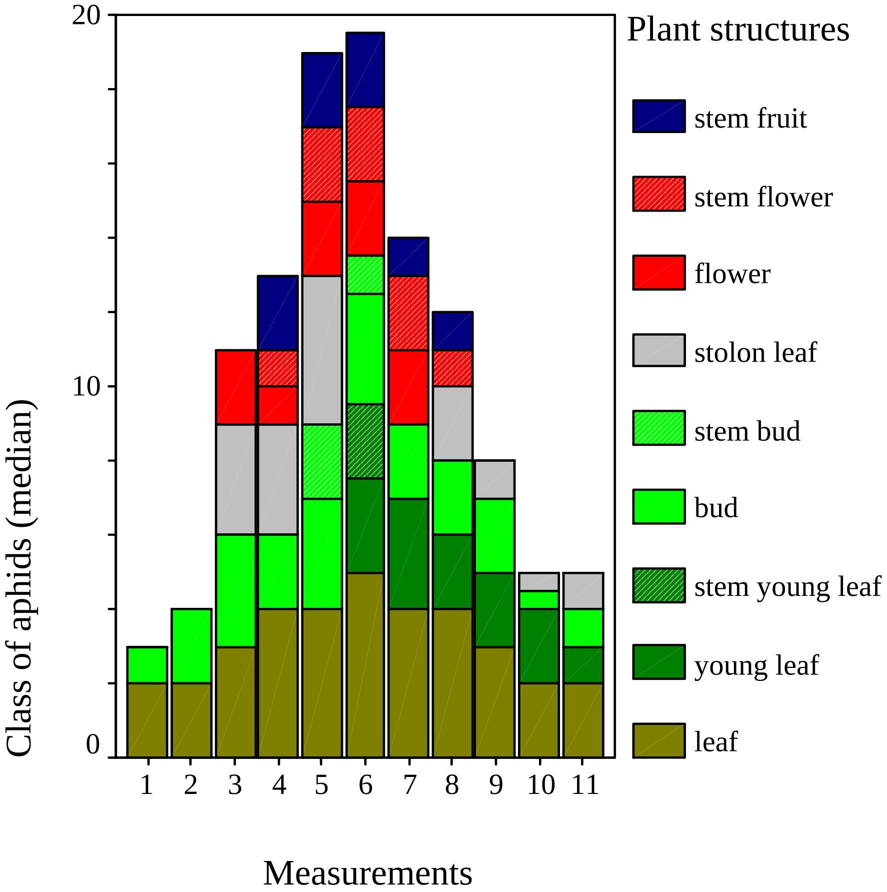

Figure 3.1. Distribution of aphids, according to the different plant structures (all compartments)

According to these results, the decision was made of dividing plant structures into vegetative and reproductive parts in order to have only the most important structures (where aphids were significantly present) being analysed.

Hence, the emergence of young leaves on the plants was continuous and a consequence of bud development: as time passed, buds grew into young leaves and new buds emerged at the crown of the plants, as shown in figure 3.2.:

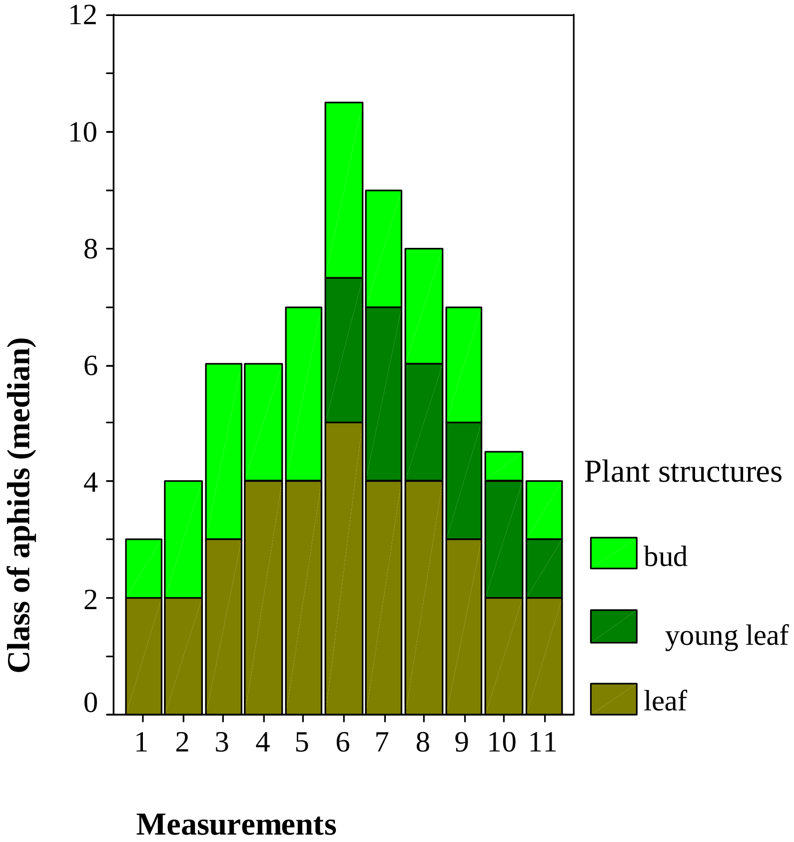

Figure 3.2. Distribution of aphids on the different vegetative plant structures

Young leaves were kept out of further discussion because the statistical analysis showed that leaves always had a higher aphid class (ANOVA, $p<0.05$) than all the other structures (immediately followed by the buds).

The same logic was applied when looking at aphid densities on the reproductive structures of the plants (Fig. 3.3.), where stolons, fruits and their stems were also excluded due to low scores or absence in some of the measurements:

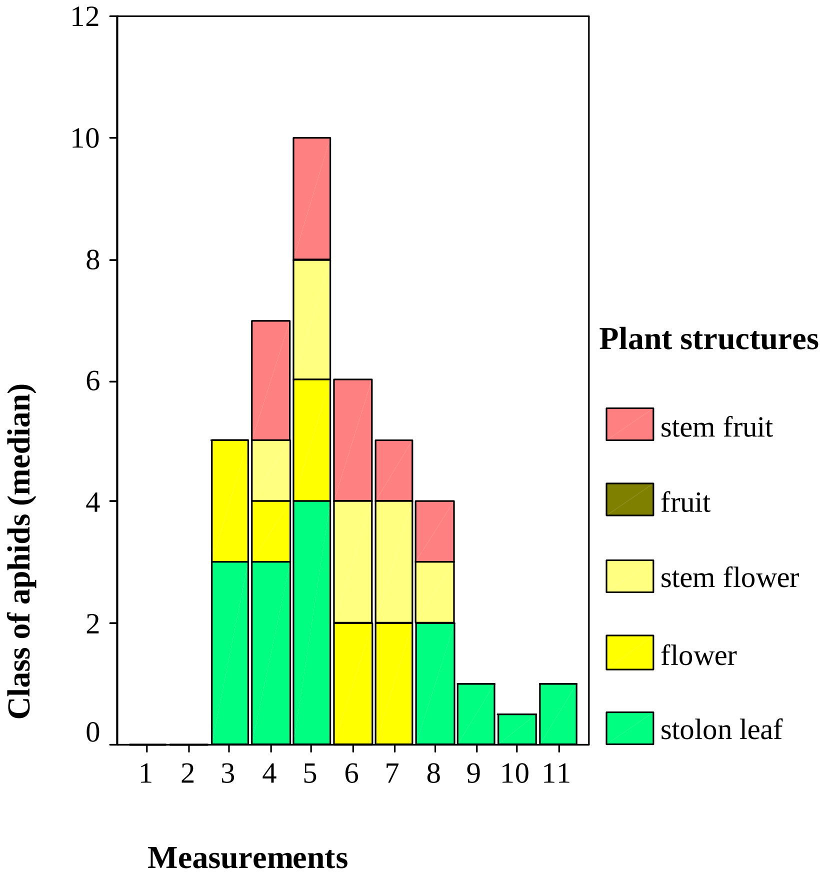

Figure 3.3. Distribution of aphids on the different reproductive plant structures

Therefore, the next sections in this chapter (and further discussion of results) include scores of aphid classes only on the four most important plant structures (Fig. 3.4.): leaves, buds, flowers and stem flowers, supported by the statistical results.

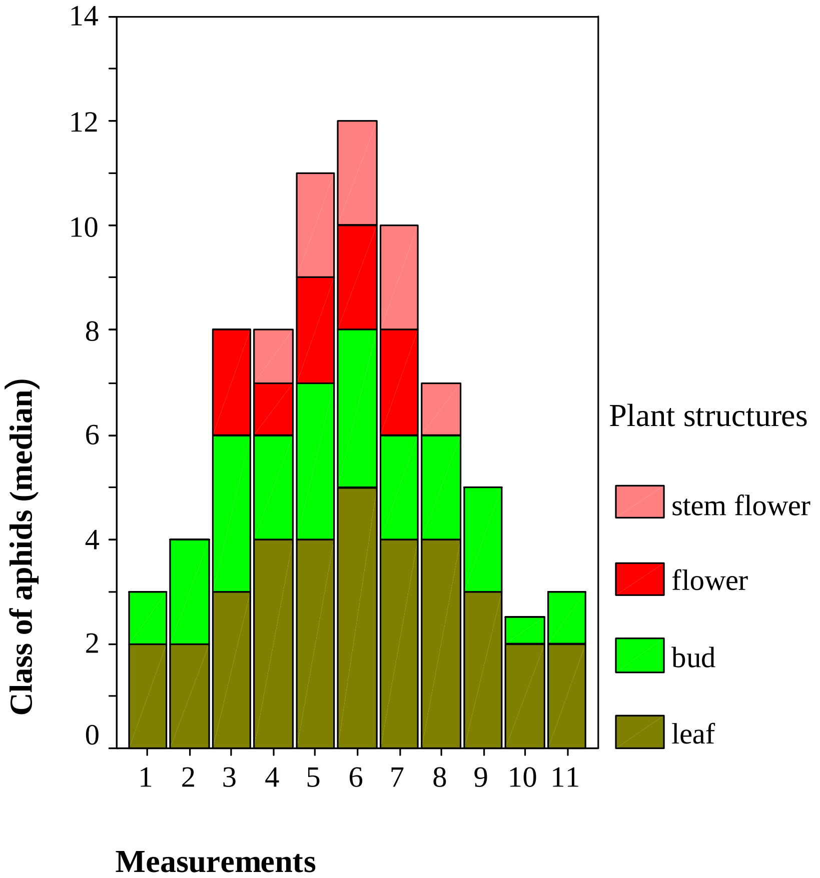

Figure 3.4. Distribution of aphids according to the most important plant structures

3.1.2. Before releasing the ladybirds

As expected, the aphid population developed in the same way in all the plants in the different compartments, i.e., spontaneous foci of aphids were spotted few weeks after transplanting of the plants and the population was allowed to develop. Scoring of aphid classes on the different plant structures started when the infestation level of the plants was still not too heavy and a typical growth of the aphid population can be seen in all compartments (which should be obvious because there was no difference between the compartments until the ladybirds were released).

Data from the eight rows was analysed separately and no differences between the rows was found, proving that the two replicates of each compartment provided similar results. Thus, data from four rows of each compartment was pooled together and analysed. No significant differences were found between wingless and winged compartments (ANOVA, F = 0.188, *p* = 0.665), as shown in figure 3.5.

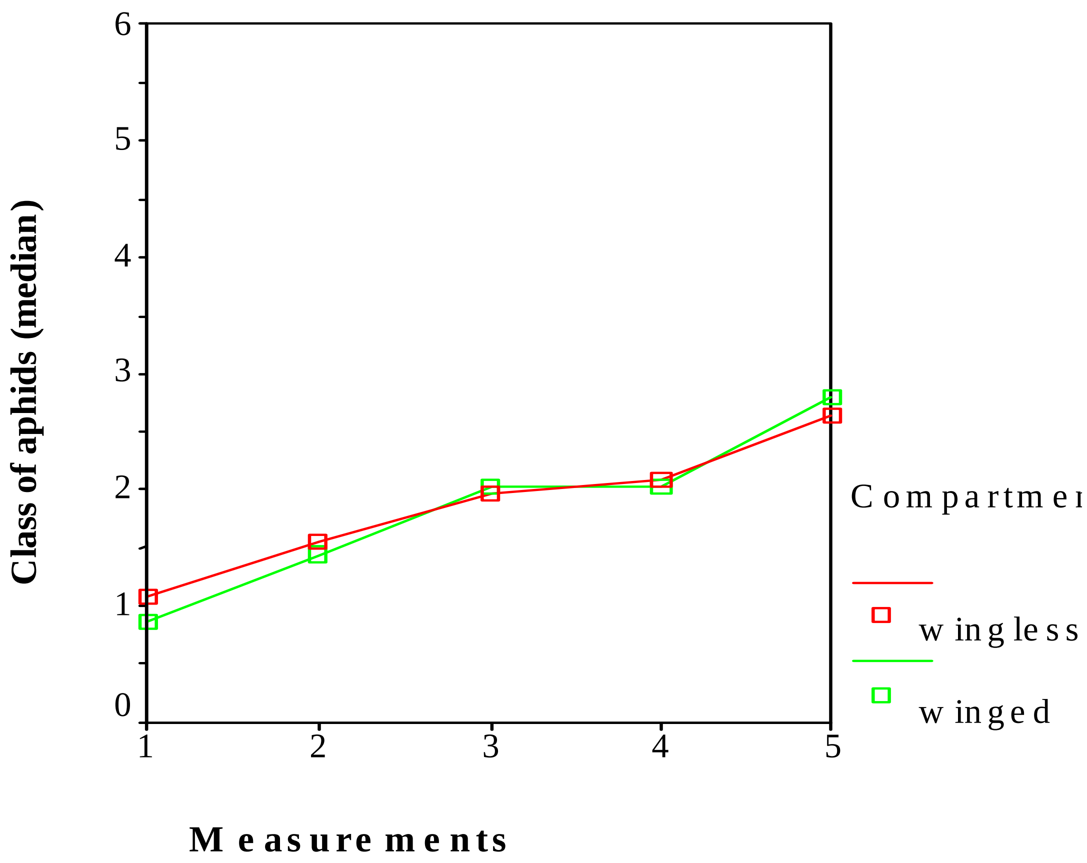

Figure 3.5. Development of aphid population before ladybird introduction: wingless vs winged compartments

There were, however, significant differences in class of aphids between all 4 plant structures, in the different measurements (fig. 3.6). Leaves always had a higher class of aphids (ANOVA, F = 122.814, *p* = 0.000) than the other three structures with an exception of measurement 3, buds always had a higher class of aphids than flowers and flower stems, flowers always had more aphids than their stems and the latter always had the lowest number of aphids.

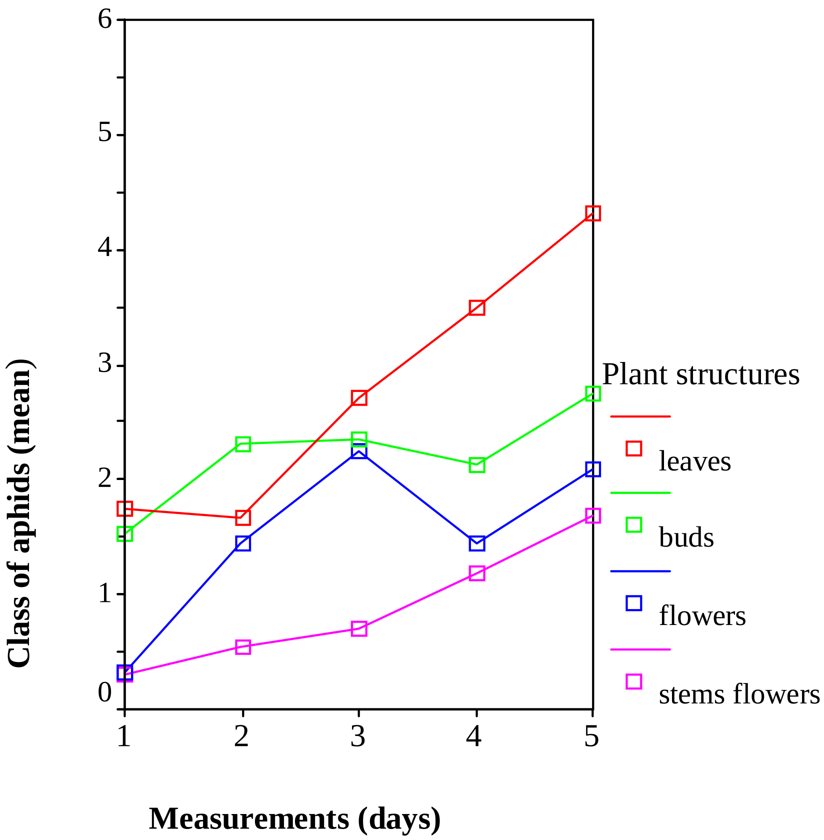

Figure 3.6. Development of aphid population according to the different plant structures, before ladybirds introduction ($p < 0.05$)

3.1.3. After releasing the ladybirds

Similar to the period before the introduction of ladybirds, the next figures show the development of the aphid population, after the release of the ladybirds, in the wingless vs. winged compartments and in the different plant structures:

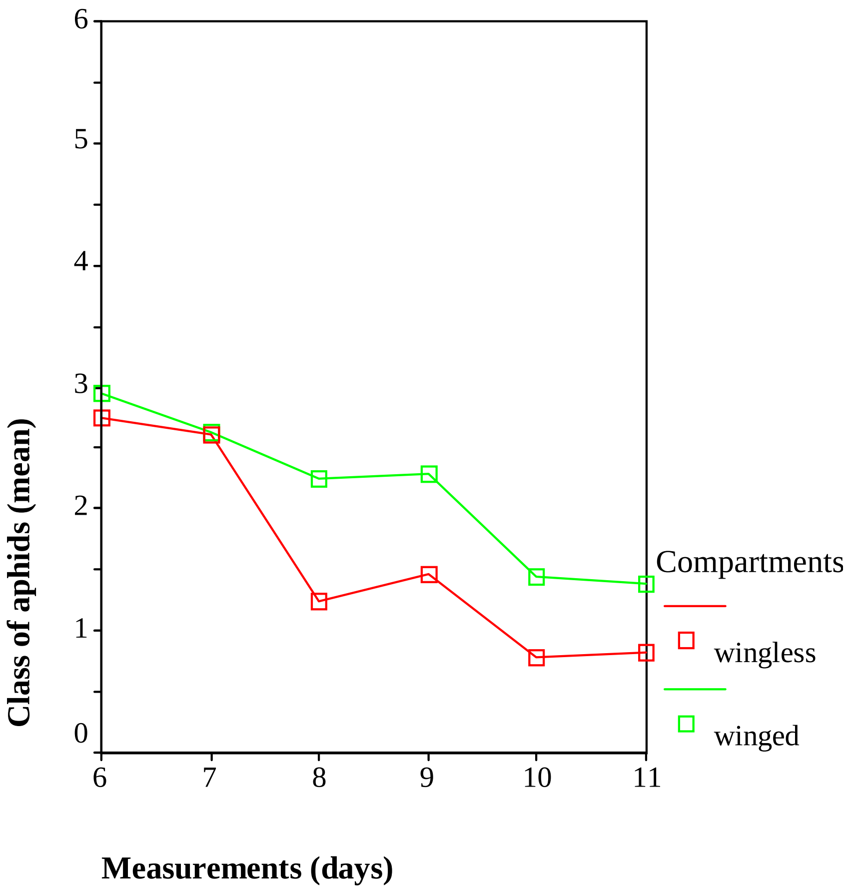

Figure 3.7. Development of aphid population after ladybird introduction: wingless vs winged compartments ($p < 0.05$)

As seen in figure 3.7, the aphid population decreased more in the compartments where wingless ladybirds were released (ANOVA, F = 7.906, $p = 0.007$) than in the compartments with winged ones.

Development of the aphid population was also significantly different between the plant structures (ANOVA, F = 76.816, $p = 0.000$): like in the measurements before the introduction of ladybirds, leaves always had a higher aphid density than the other three plant structures (Fig. 3.8). Buds had higher aphid densities than flowers (with an exception on measurement 10) and than their stems (with exceptions on measurement 8 and 10). In contrast to the period before the release of ladybirds, flowers had the lowest aphid densities instead of their stems.

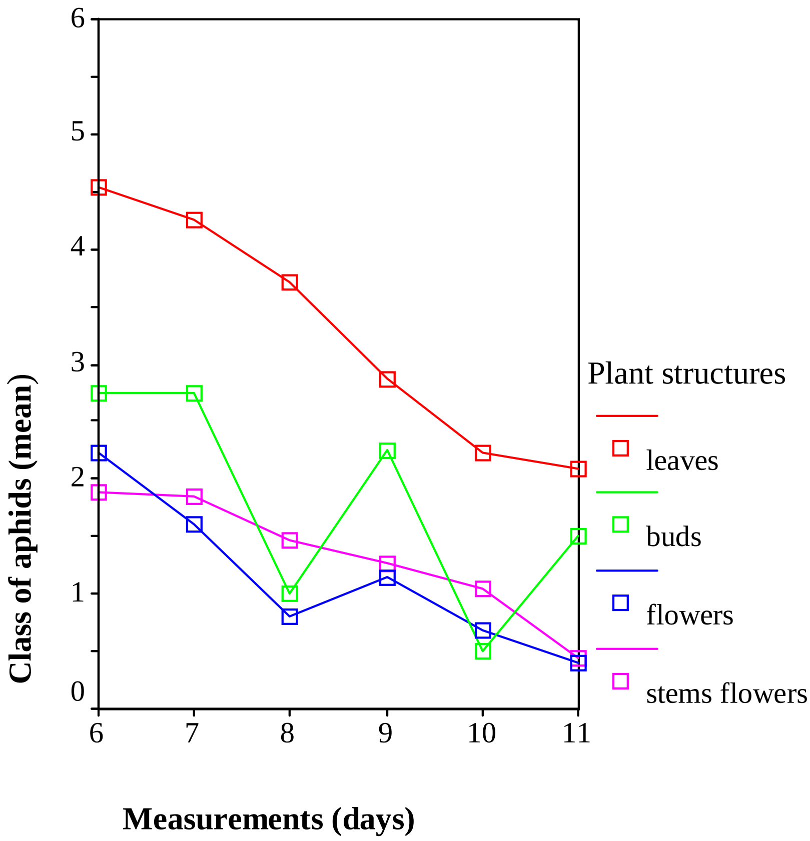

Figure 3.8. Development of aphid population according to the different plant structures, after ladybird introduction ($p < 0.05$)

3.1.4. Aphid population development

Figure 3.9 shows the development of aphids throughout the entire experimental period: the similar initial growth of aphids in wingless and winged compartments and the clear difference in the reduction of aphid numbers after the introduction of ladybirds is shown (notice that in all measurements after the ladybirds were released, scores of aphids on wingless compartments were always lower than in the winged ones).

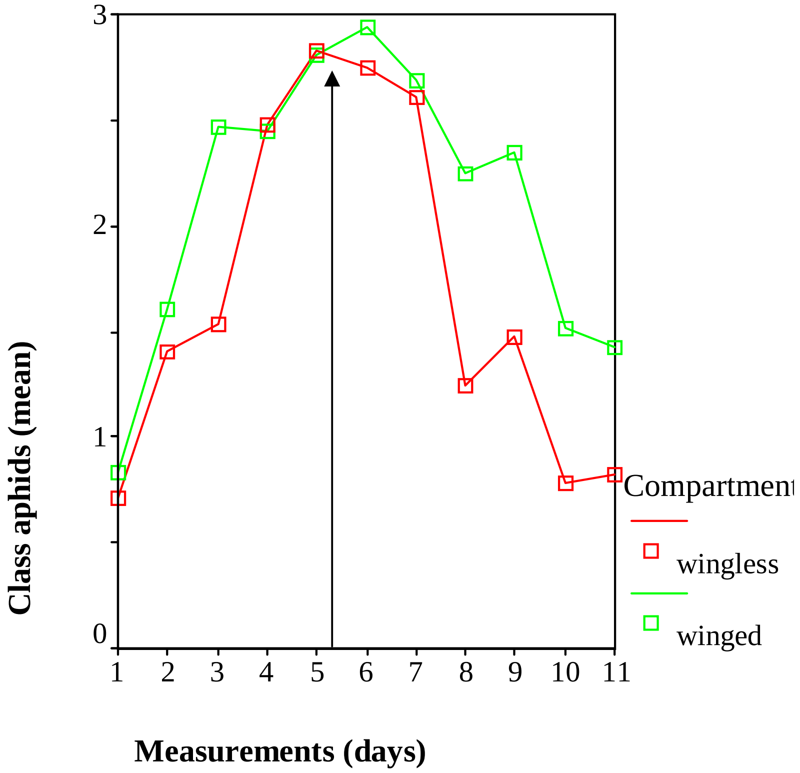

Figure 3.9. Development of aphid population (average scores of all plant structures) during the entire experimental period: wingless vs winged compartments (arrow indicates introduction of ladybirds)

When analysing the results of this experiment, several figures were obtained where the similarities between different rows within the same compartment are clear, for all the four plant structures. The statistical analysis showed no significant differences between these rows and for sake of keeping the reader interested in these results, only an overview of aphid distribution on all four plant structures (Fig. 3.10) and on leaves (Fig. 3.11) is shown here:

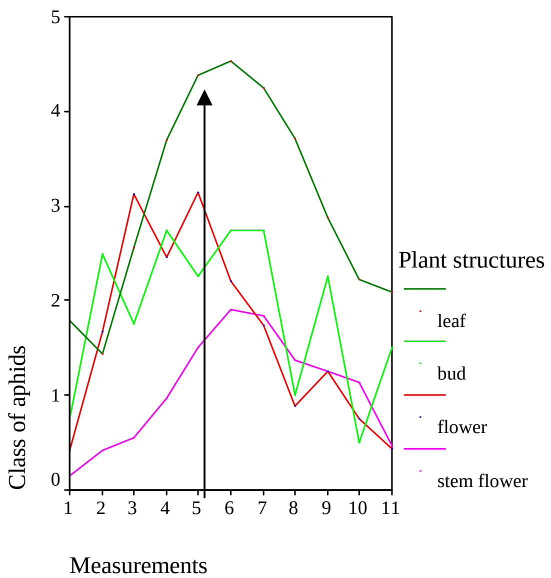

Figure 3.10. Development of aphid population on all four plant structures, during the entire experimental period (arrow indicates introduction of ladybirds)

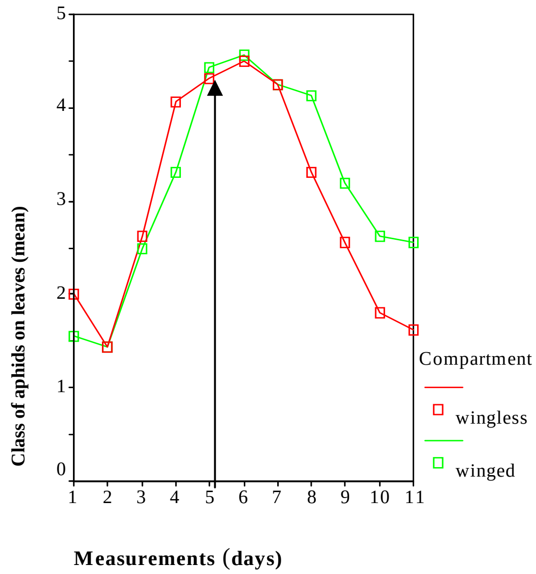

Figure 3.11. Development of aphid population on leaves, during the entire experimental period: wingless vs winged compartments (arrow indicates introduction of ladybirds)

As seen in figure 3.11, aphid classes on leaves were consistently lower in the wingless compartments, after the ladybirds were placed on the plants. It is also interesting to see in the next figure how the aphid population developed when the different compartments were analysed separately:

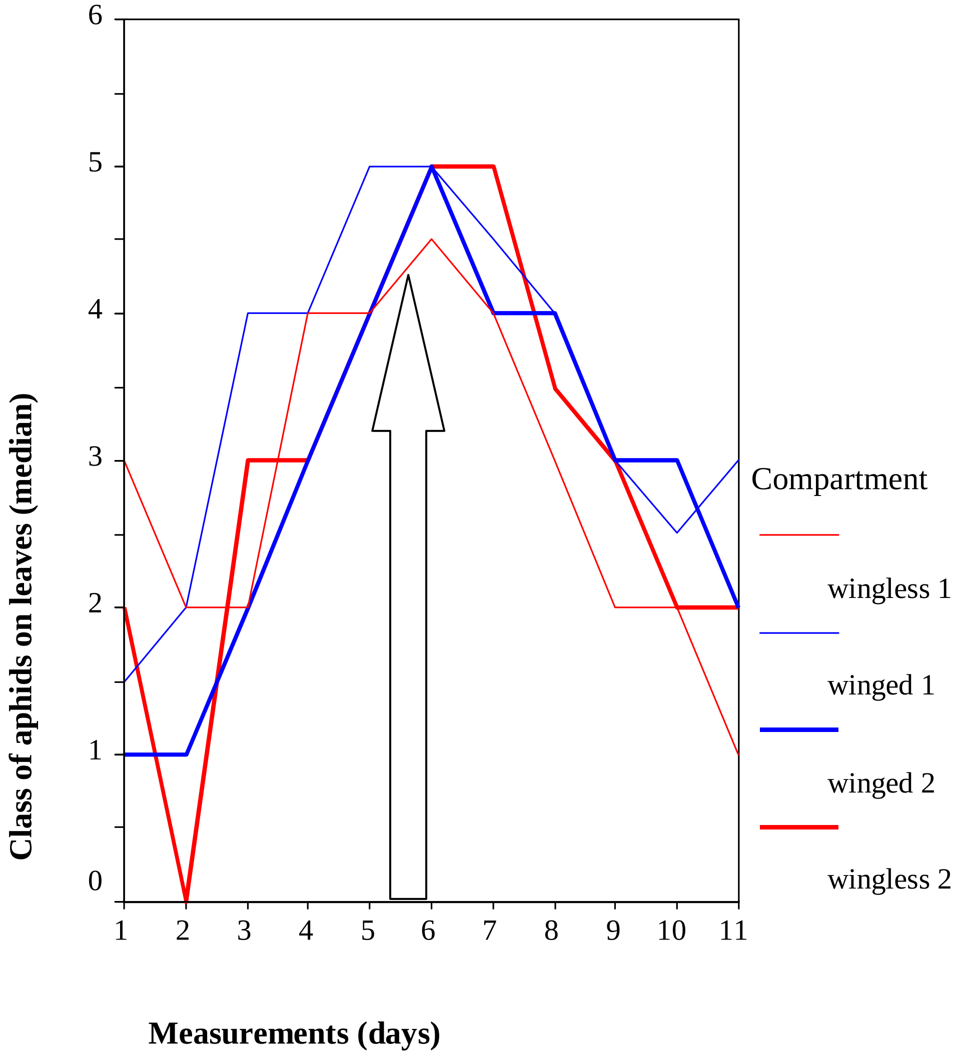

Figure 3.12. Development of aphid population on leaves, during the entire experimental period: individual compartments (arrow indicates introduction of ladybirds)

Again, all aphid scores after ladybird release were consistently lower in the wingless compartments (with a marked different slope) than in the winged ones (Fig. 3.12.).

Additionally, some insects, predators and parasitoids of aphids, were occasionally found in all compartments.

3.1.5. Ladybird population development

The average lower number of aphids in wingless compartments shown in the previous sections, is associated with larger numbers of ladybirds found on the plants in the referred compartments and larger number of winged ladybirds found around the compartment (hence, lower numbers found on the plants).

There were no significant differences (χ^2, p> 0.05) between wingless and winged compartments, when the measurements were tested individually (except for measurement 3, as shown in appendix XI). However, the number of ladybirds found on the scored plants, in wingless compartments was consistently higher than in winged compartments throughout the measurements, showing a trend equal to one of winged ladybirds found around the compartments (also always higher in winged than in wingless compartments), as shown in the following figure:

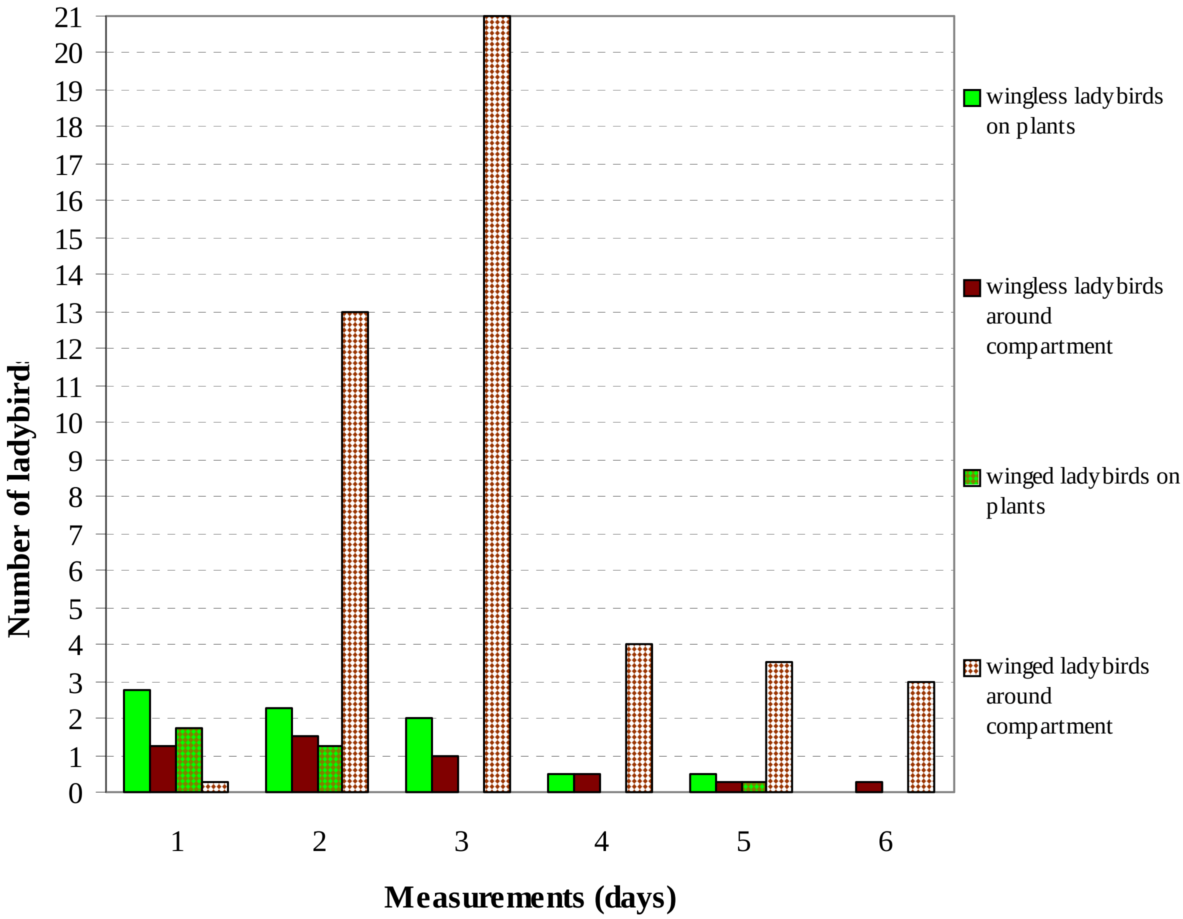

Figure 3.13. Ladybirds found on the plants and around the compartment (wingless vs winged)

Because of this obvious result and because numbers of ladybirds of each individual measurement were low, the scores from the different measurements were pooled for further analysis and to check if indeed wingless ladybirds do stay on the plants and winged ones fly away from them. It does not matter that number of found ladybirds was cumulative (these are alive ladybirds and it is possible that the same individual was scored more than once, in different measurements) and it is safe to assume that scores from different measurements were independent from each other. The chance of finding one individual on a plant, or around the compartment, in one measurement was the same as in the following(s) measurement(s) as the behaviour of the ladybirds can be predicted to be the same in one and other(s) measurement(s). As the plants were always infested with aphids during the whole experimental period, it is normal that the beetles would show a high mobility, both in the plants and around the compartments, in all the measurements.

When the total numbers of found ladybirds were analysed, again the trend is very clear. Numbers of wingless ladybirds found on plants are significantly higher than winged ladybirds on plants and number of winged ladybirds found around the compartment higher than those of wingless ones (χ^2, Pearson = 21.155, p = 0.000).

Figure 3.14. Number of wingless and winged ladybirds, found on plants and around the compartments

Figure 3.14 shows that from the total numbers, 21% were wingless ladybirds and 79% were winged. Within the wingless group, 62% were found on the plants and 38% around the compartment. Within the winged group, only 6% were found on the plants whilst 94% were around the compartments.

The few observations on oviposition and larval development indicated that some reproduction took place in the ladybird population, in all compartments but more eggs were found on the plants in wingless than winged ones.

3.2. BEHAVIOURAL EXPERIMENT

The results showed significant differences (χ^2, $p < 0.005$) between wingless and winged ladybirds, regarding their location (on the plant or around the cage), for all the scores (except for the first score, as seen in appendix XII a).

Figure 3.16 shows the numbers of ladybirds that were found on the plant and on the cage, up to 8 hours after release. Even though some wingless ladybirds left the plant, the majority of them stayed on the plants throughout the 8 hours of the experiment. In contrast, winged ladybirds remained on the plants in the first score but in the next one, their numbers were already lower and decreased rapidly every hour, until all ladybirds had left the plant within six hours after release.

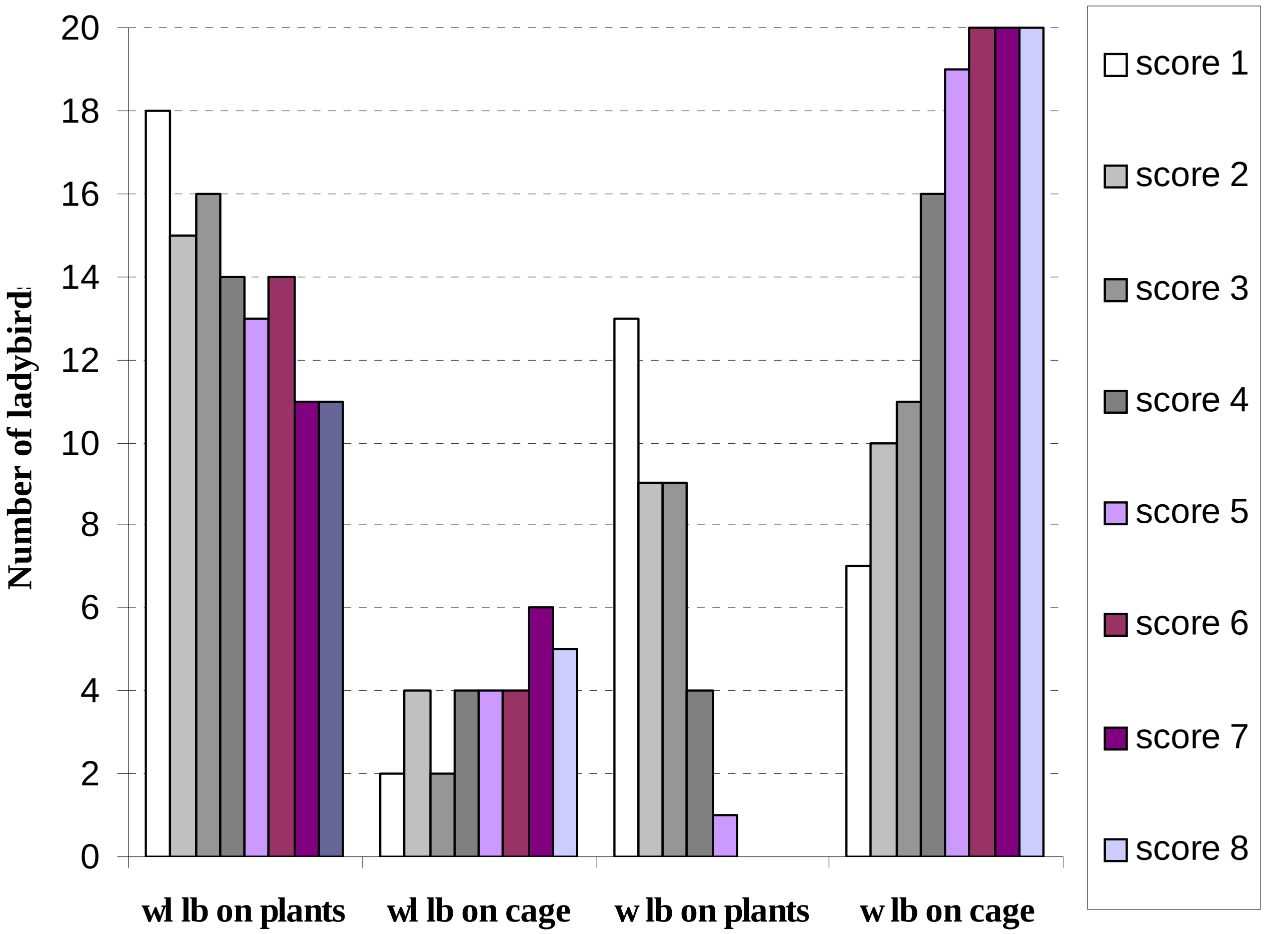

Figure 3.16. Number of ladybirds per score (average of the 10 sets), on the plant or around the cage

Figure 3.17 shows a similar result to the one of figure 3.14 (number of wingless and winged ladybirds, found on the plants and around the compartments, in the field experiment): there is a significant difference (χ^2, Pearson = 93.403, p = 0 .000) between winged and wingless ladybirds regarding their location (on the plant or around the cage). From the total number of observed ladybirds, 47% were wingless and 53% winged (some of the ladybirds were not found in some of the scores, mostly wingless ones, and that explains these percentages). Within the wingless ones, 78% were found on the plant and only 22% around the cage and within the winged group, 23% were found on the plant whilst 77% spent most of their time around the cage.

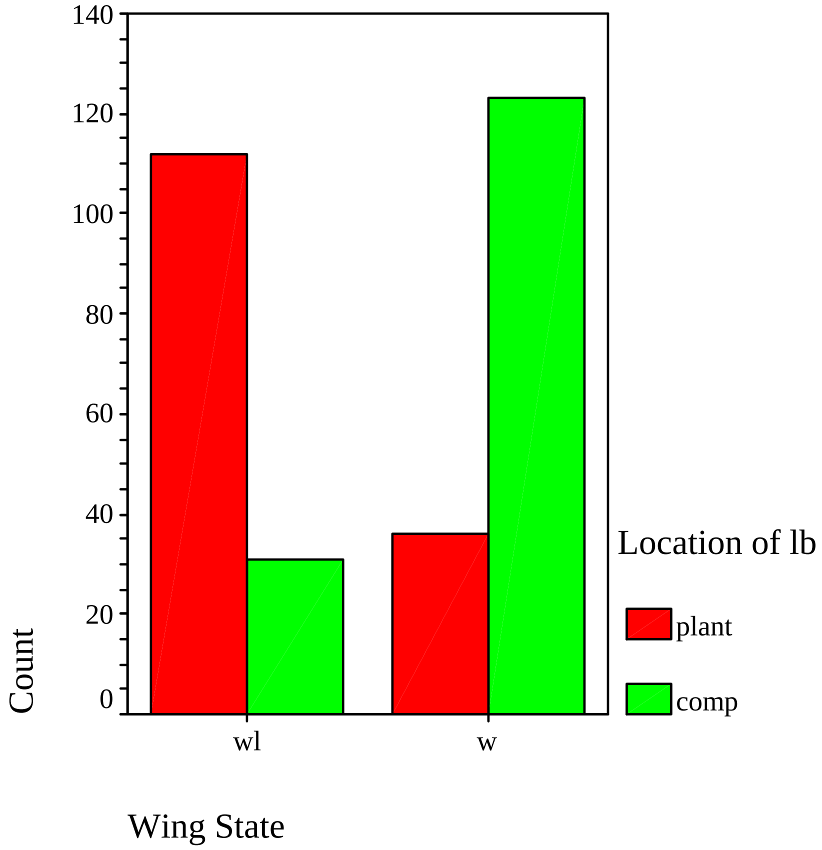

Figure 3.17. Number of ladybirds on the plant or around the cage (sum of all scores from the 10 sets)

From the total number of ladybirds found on the plant, almost 76% were wingless and from the total number found around the cage almost 80% were winged.

Because these observations did not replicate exactly the conditions of the polytunnel (where a much lower number of beetles per plant was used), another test was done this time comparing average of all the scores, of each set of ladybirds observed. This analysis was carried out just to prove that no matter the number of individuals used to control the aphids, the results are always concordant and conclusive: wingless ladybirds stay on the plant for longer time and winged ones fly away almost immediately after release.

Thus, the number of ladybirds found on the plant and around the cage, of all the scores, was averaged for each set and then compared. Figure 3.18 shows the number of wingless and winged ladybirds, found on the plants and / or around the cage, for each of the 10 sets:

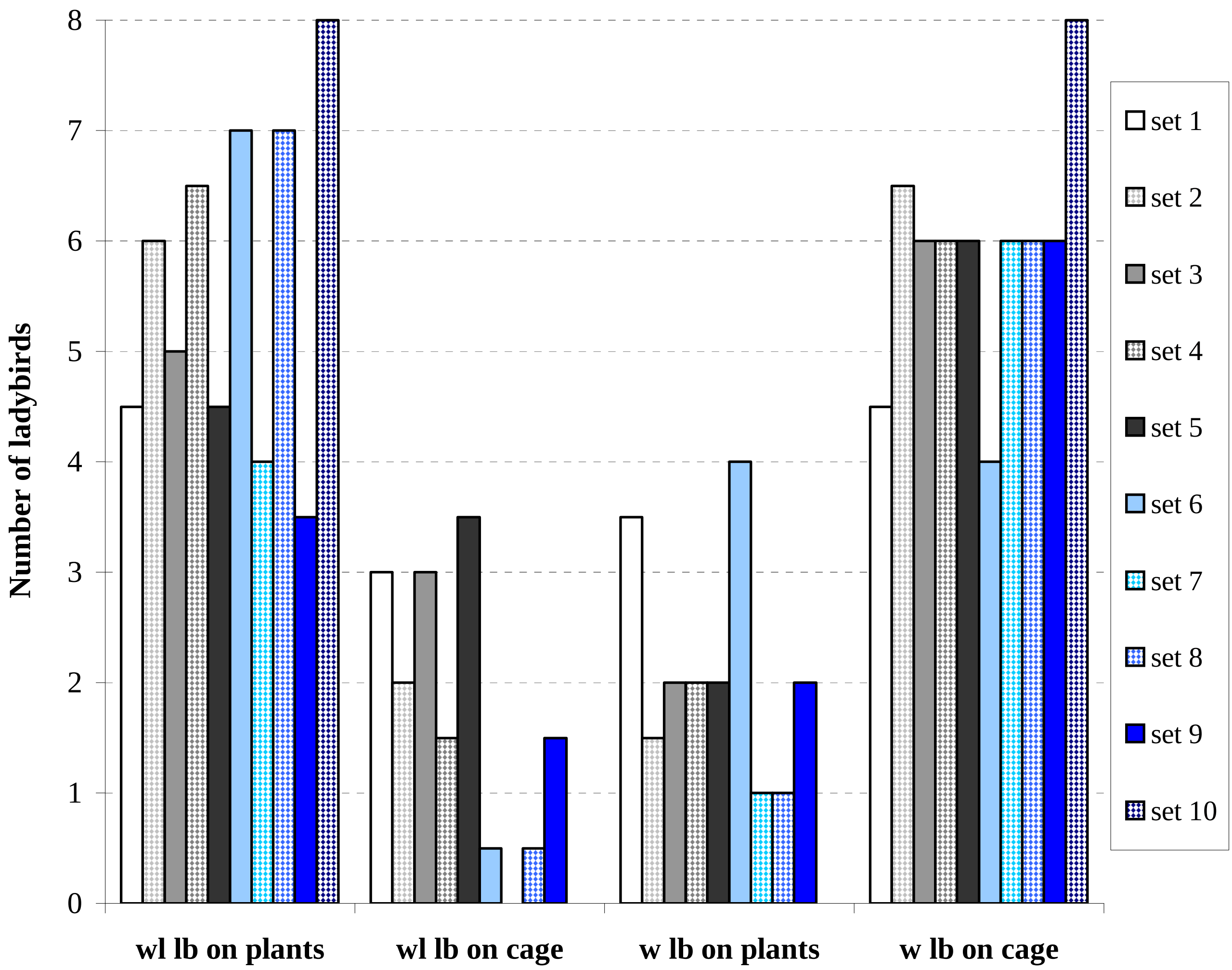

Figure 3.18. Number of ladybirds per set (average of the 8 scores), on the plant or around the cage (bars with triangular pattern indicate significantly different sets, as seen in appendix XII b)

The difference between wingless and winged ladybirds, regarding their location, was significant in 5 of the 10 sets (appendix XIII b) showing that maybe there was an effect of the individuals used (remember that this test compared different sets of ladybirds). Those sets are shown in the figure with a triangular pattern filling (sets 2, 4, 7, 8 and 10) whilst the ones where no significant difference was found are filled only by a colour.

For each set (regardless the differences between sets), again a trend is found: the numbers of wingless beetles found on the plant are always higher than those of winged ones, as well as higher than wingless ones found on the cage. Similarly, the numbers of winged ladybirds found on the cage are always higher than those of the wingless ones, as well as higher (except in set 6, where the numbers are equal) than those of winged ones found on the plant.

When the results from all the sets were pooled and the number of wingless or winged ladybirds averaged for all the scores, the same result is obtained confirming that wingless ladybirds stay on the plant and winged ones fly to the cage. Obviously, this difference is caused by those sets were a higher number of wingless beetles remained on the plant for a longer period of time and a higher number of winged ones remained somewhere around the cage. Figure 3.19 shows a result similar to the one of figure 3.17 (number of ladybirds on the plant or around the cage: sum of all scores from the 10 sets): there is a very strong significant difference (p = 0 .000) between winged and wingless ladybirds regarding their location. As when average of the scores were pooled together, 48% of the ladybirds observed were wingless and 52% were winged (again, some of the ladybirds were not found in some of the sets and most were wingless ones). Within the wingless group, 78% were found on the plant and only 22% around the cage and within the winged group, 24% were found on the plant whilst 76% spent most of their time around the cage. It can also be pointed that from the total number of ladybirds found on the plant, almost 75% were wingless and from the total number found around the cage almost 79% were winged.

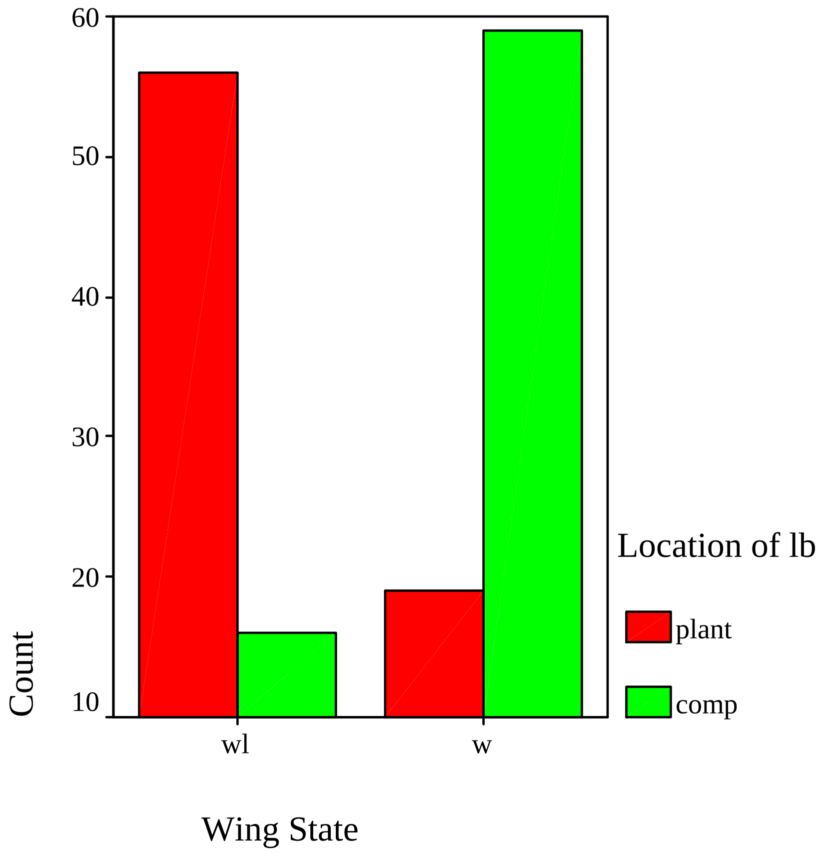

Figure 3.19. Number of ladybirds, on the plant or around the cage (sum of all sets)

4. DISCUSSION

4.1. FIELD EXPERIMENT

As expected, the aphid population followed a typical growth curve consistently similar in all compartments, before the introduction of the beetles (Fig. 3.5), with no differences between the wingless and winged compartments. This growth curve started to decline after the release of the ladybirds, with a clear marked difference between wingless and winged compartments.

Aphid classes were significantly lower in the compartments where wingless beetles were present than in the ones were winged beetles were (Fig. 3.7), confirming that wingless ladybirds are more efficient at controlling aphids, as the predation rate by the beetles should be the same, whether they are winged or not. This is supported by the higher number of wingless beetles found on the plants and the higher number of winged ones found around the compartments (Fig.3.13).

As not all the released beetles were traced back, but still more were found on the plants, in the wingless compartments than in the winged ones, it is possible that some of the winged flew away from the compartments or hid themselves in small holes. Still, the winged beetles were not found on the plants which is consistent with the higher aphid classes scored on those compartments.

It is also possible that the decline in the aphid population after introduction of the beetles was affected by the presence of other insects, predators and parasitoids of aphids. This was not extensively studied in this experiment since it was not considered to affect the results because these insects were present in all compartments so, if they did help reducing the aphid population, this happened in a similar way for both winged and wingless compartments.

Aphidophagous ladybirds have already been considered to be not such efficient control agents because of the lack of synchrony between their developmental time and the one from their preys, which is faster (Dixon *et al.*, 1997). It seems that aphid populations are allowed to develop faster because the beetles can not cope with their increase as they take longer to develop. In order to increase their effectiveness, ladybeetles need to synchronize their reproduction with the early development of the aphid population (Hemptinne *et al.*, 1992). However, the experiment described in this report was not designed to study the synchronization between ladybird and aphid populations and its consequence on aphid control, but to measure the efficiency of the beetles in remaining on the plants. Still, mating, oviposition and larval development were taken into consideration and roughly checked indicating that some reproduction did took place.

Nevertheless, winged ladybirds are used as biological control agents of aphids in greenhouses, and are known to rapidly fly away from the plants, making it rather difficult to establish populations (Hämäläinen, 1977). It seems that the effectiveness of these beetles should be evaluated considering larval voracity instead of adult control because adults tend to fly out from the greenhouse, especially during the pre-oviposition period (Gurney & Hussey, 1970). The results of the field experiment confirm this, as the winged beetles were found less on the plants and the presence of aphids on the plants was higher than for the wingless ones.

4.2. BEHAVIOURAL EXPERIMENT

The results of the behavioural observations of the beetles on a plant inside a cage confirmed the field results: wingless ladybirds do remain on the plant and winged ones fly away (Fig. 3.17).

Even though these observations did not replicate exactly the field conditions, statistical analysis of the results proved that regardless the number of individuals used, wingless ladybirds remain on the plants for longer periods and winged ones fly away. It can be discussed that a higher number of beetles present per plant, as used in these behavioural observations, might have affected the results by affecting the beetle's behaviour and this is why comparing the different scores (location of the beetles at different time moments) seemed more reliable than comparing sets of ladybirds (sets did not consist of the same individuals and there was a difference in their age).

It seems that wingless ladybirds spend more time foraging the plant than winged ones, because they don't fly. Although no statistical results were presented, a preliminary analysis regarding the beetles activity (see table 2.6), seem to indicate that wingless individuals spend considerably more time walking on the plant while winged ones share their time between walking and remaining quiet on the cage. It is possible that the lamp placed directly above the cage (and thus heating up the top glass) and the paint of the wooden frame have somehow attracted the winged beetles. This remains unclear but it is clear that they do fly away from the plant even if many aphids are available.

4.3. GENERAL

Regardless these encouraging results, more research should be done with bigger samples, both for the field and the behavioural experiments. In addition to checking if wingless beetles remain on the plants and winged ones fly away, reproductive features should also be more thoroughly studied. Effectiveness of these beetles as biological control agent of aphids is related not only to their residence time on the plants but also to their voracity and ability to reproduce and develop at a proper rate (synchronized with the prey population), so that populations can be established successfully. Hence experiments on oviposition rates, developmental times and larval and adult voracity for both wing states should be considered.

5. CONCLUSIONS

Measuring the efficiency of the wingless ladybirds as biological control agents of aphids, by monitoring both populations in a field trial and studying permanence time on a plant in the laboratory showed that:

1. In field conditions, wingless ladybirds were more efficient controlling the aphid population than the winged ones: aphid numbers were consistently lower and number of ladybirds found on the plants higher, in the wingless compartments than in the winged ones.

2. Regardless the number of individuals used, time scale or surrounding environment, the behavioural observations support the field results: wingless ladybirds remain on a plant for longer periods whilst winged ones quickly fly away.

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APPENDICES

APPENDIX I. REARING METHOD FOR *ADALIA BIPUNCTATA*

General

- Rearing of the ladybirds (lb) was done **3 times per week** (Mondays, Tuesdays and Fridays), inside a climate chamber at 20 ºC and 60 % humidity
- The wingless stock resulted from crossings with wild ladybirds (winged) and posterior selection, meaning that this rearing included:

Designation of ladybird type and morph	**Genotype**
wingless	ee
winged	EE or Ee
typica	tt
melanic	mm or mt

Examples of crossings
eett x eett
eett x eemt
eemm x Eett
eemm x Eemt
eemt x Eemm

Ladybirds were fed with:

- ***Ephestia*** eggs, stored at -20 ºC and placed in a fridge 1 day before used
- **Bee pollen**, stored at -20 ºC and smashed on the moment lb were fed
- **Food waste and dead lb** were frozen at -20 ºC, before being thrown away

Adults

- About **30 ladybirds** (crossings of 15 ♂ and 15 ♀) were placed in one petridish (12 cm ∅)
- Crosses were **labeled** by genotype ♂ x genotype ♀ and date
- A white paper was placed on lid of petridish for **egg laying:**
  a. **paper changed** when reasonable number of eggs on it
  b. **paper and petridish changed** when reasonable number of eggs on both (meaning that adults were removed to a new dish with a new paper)
- Paper with eggs was cut into smaller pieces and placed inside a new dish or in the original one (according to the number present and if dish had to be replaced or not); petridishes **labeled with** genotype of offspring (when homozygotes) **or** with genotype of parents (when offspring contained many different genotypes). Ex: offspring from eett x eett was directly labeled as eett but offspring of eett x Eemt was labeled with parental genotype and separated by phenotypes only after adult emergence (as chances of getting one of the possible phenotypes was variable)
- **Adults were removed** to new petridishes when too much waste was present, even if no eggs had been laid
- **Dead adults** were always removed
- **Fed with** 5 mg *Ephestia* eggs per lb/ day and bee pollen was given *ad libitum*
- when number of ladybirds was reaching 10-15, adults from different petridishes were **mixed up** to 30 again

Larvae

- About **30** were kept in one petridish (12 cm ∅)
- **Dead ones** were always removed
- **Divided** over more petridishes as soon as big enough to handle with a brush
- **Rearranged** over more petridishes according to size, when this was varying a lot inside one petridish
- **Old food** was **removed by** tapping the edge of the petridishes **or** larvae were moved to a new petridish when too much waste was present (about once a week)
- **Fed** with *Ephestia* eggs (same amount as for adults but adjusted for larval instar)
- **Moved** to new petridishes when part of the group was a prepupa or pupa

Pupae

- **Old food** (when present) was **removed** by tapping edge of petridish on the table

New adults

- Adults emerged from pupae were **removed**, **sexed** and **separated by phenotypes** (wingless or winged, melanic or *typica*)
- **Placed** in new petridishes, **labeled** with genotype

APPENDIX II. POLYTUNNEL MAP (FIELD EXPERIMENT)

WL 1 and WL 2 are wingless compartments
W 1 and W 2 are winged compartments
Numbers inside rectangles indicate trays, with 6 plants each

• Trays with plant where aphids were scored, before ladybird introduction and with plants where beetles were released
* Trays with plant where aphids were scored, after ladybird introduction

APPENDIX III. PEST MANAGEMENT PROGRAMME (FIELD EXPERIMENT)

The bio control of possible pests was done according to a strict protocol provided by Koppert BV but, generally, the following products were used :

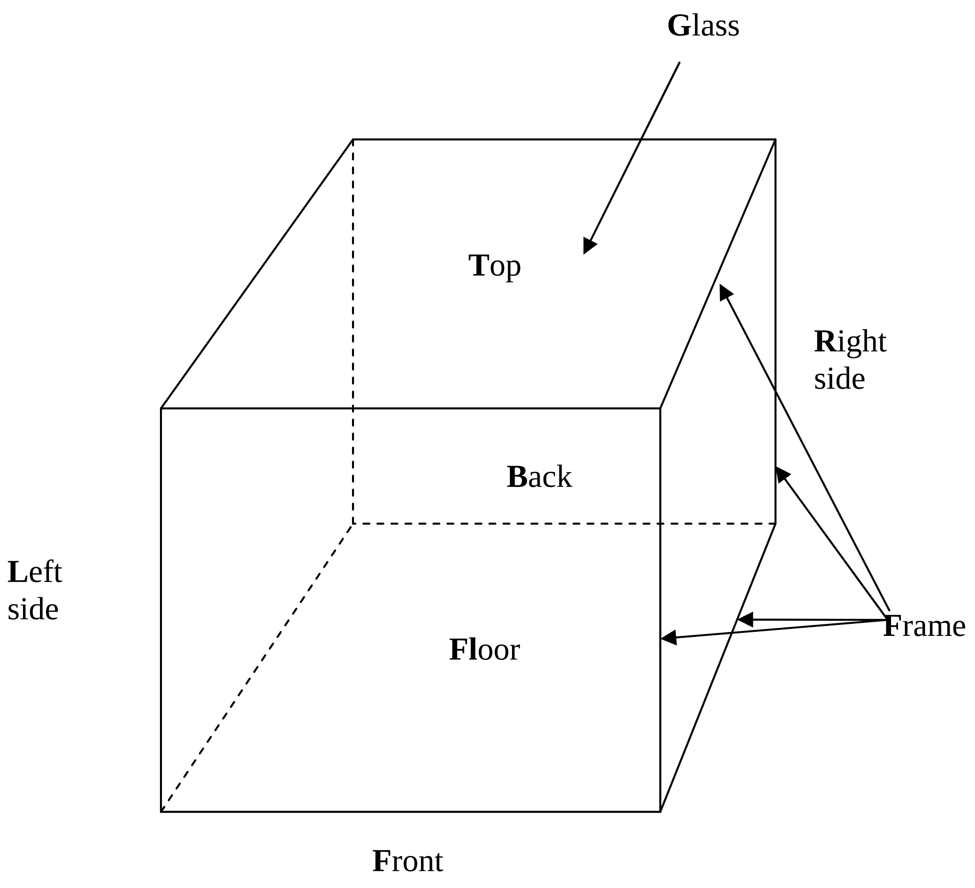
- Bumble bess, for pollination of the plants
- SPIDEX, to control spider mites, applied every 2 weeks
- THRIPLEX-PLUS, to control thrips, applied every 6 weeks
- ENERMIX, to control white flies, every 2 weeks

Appendix IV. Time schedule (Field experiment)

Month	Trial week	Measurement days	Date	Activity
July	1		22	Placement of strawberry plants in the trays, on the polytunnel Collection of winged ladybirds in the wild
	2			
August	3		05	Collection of winged ladybirds in the wild Natural outbreak of aphids
	4			Release of pollinator agents
	5	1	16	**Start Field Experiment:** monitoring aphid population (1 plant per tray, in every tray)
		2	20	Monitoring aphid population
	6	3	24	Monitoring aphid population
		4	28	Monitoring aphid population
	7	5	31	Monitoring aphid population
September			01	**Introduction of winged & wingless ladybirds**
		6	03	Monitoring ladybirds & aphids population, together in the same plant (1 plant per tray, in every two trays)
	8	7	07	Monitoring ladybirds & aphids population
		8	10	Monitoring ladybirds & aphids population
	9	9	14	Monitoring ladybirds & aphids population
			15	Cleaning of the floor in all compartments (too much water and plant material present) Collection of aphids and other insects, from all compartments
			16	Identification of the aphids and other insects collected
		10	17	Monitoring ladybirds & aphids population
	10	11	22	Monitoring ladybirds & aphids population: **End Field Experiment**
	11		27	Collection of remaining ladybirds and counting of larvae

APPENDIX V. STRAWBERRY PLANT STRUCTURES (FIELD EXPERIMENT)

APPENDIX VI. CAGE (BEHAVIOURAL OBSERVATIONS)



APPENDIX VII. CLEANING OF PLANT (BEHAVIOURAL OBSERVATIONS)

- The plant (with few number of stems but higher number of young leaves and buds) was collected from the polytunnel, where the field experiment took place, on the 05.10.04 (about one month before the behavioural observations were done)
- After transportation to the Wageningen laboratory (where the observations were carried out), the plant was covered with a black plastic bag and white tissues were placed beneath the roots and around them (beneath crown of plant)
- The older leaves were removed and the oxygen inside the bag removed
- After closing it, a CO_2 flow (3 bar/ min) was allowed to enter the bag for about 1-2 min, after which the bag was removed
- Inspection of the plant for live organisms was done, followed by tapping and some insects dropped dead (aphids mainly) but exact counts were not done
- The plant was placed in a pot, with new soil (strawberry substrate), watered and fed with nutrients
- The plant was kept at room temperature, until the first day of observations

APPENDIX VIII. REARING APHIDS (BEHAVIOURAL OBSERVATIONS)

- An unknown number of aphids was collected from the plants in the polytunnel (when the plant for the behavioural observations was collected), by cutting the plant part where they were or with a brush, directly into a plastic box
- In the same day, 3 extra plants were collected and cleaned later, as described in appendix VII
- Initially, the aphids were reared in 7 cm Ø petridishes, with a 1- 2 cm high layer of nutritious agar (10%) and a strawberry leaf, cut from the clean collected plants (with the same 7 cm Ø)
- Because the number of dead was increasing (the aphids appeared to be have been parasitised) and reproduction was not overcoming death, the healthy adults were chosen and their offspring separated into petridishes
- After placing the cleans plants inside a mesh sleeve, the healthy offspring (in several different stages) was placed on them, with a brush
- Healthy adults were removed frequently and placed on the plant for the behavioural observations, when necessary (always, when below 20 aphids)

APPENDIX IX. SCORES (BEHAVIOURAL OBSERVATIONS)

Score number	Time (hours)	Observation
	9.00	Placement of the ladybirds on the leaves
	9.00 - 9.30	After release, observation for ½ hour continuously
1	10.00	Location and behavioural scores
2	11.00	Location and behavioural scores
3	12.00	Location and behavioural scores
	12.00 - 12.30	After hours, observation for ½ hour continuously
4	13.00	Location and behavioural scores
5	14.00	Location and behavioural scores
6	15.00	Location and behavioural scores
7	16.00	Location and behavioural scores
8	17.00	Location and behavioural scores + removing and freezing of the ladybirds

APPENDIX X. ANOVA TABLES (FIELD EXPERIMENT)

Before introduction of ladybirds

Source	F	Sig.
Wing State	0.188	0,665
Plant structure	122.814	0,000

After introduction of ladybirds

Source	F	Sig.
Wing State	7.906	0,007
Plant Structure	76.816	0,000

APPENDIX XI. CHI-SQUARE TEST RESULTS FOR LADYBIRD NUMBERS (FIELD EXPERIMENT)

Measurement	**Phi** (approx. sig.)	**Fisher's Exact Test** exact sig. (1-sided)
1	0.439	0.667
2	0.043	0.108
3	0.000	0.011
4	0.121	0.333
5	0.025	0.200
6	0.046	/

Appendix XII a. Chi-square test results for ladybirds' location- scores comparison (Behavioural observations)

Score	**Phi** (approx. sig.)	**Fisher's Exact Test** exact sig. (1-sided)
1	0.058	0.064
2	0.044	0.046*
3	0.004	0.005*
4	0.000	0.000*
5	0.000	0.000*
6	0.000	0.000*
7	0.000	0.000*
8	0.000	0.000*

* Significantly different scores

Appendix XII b. a. Chi-square test results for ladybirds' location- sets comparison (Behavioural observations)

Set	**Phi** (approx. sig.)	**Fisher's Exact Test** exact sig. (1-sided)
1	0.457	0.399
2	0.030	0.044*
3	0.131	0.157
4	0.030	0.044*
5	0.201	0.218
6	0.106	0.141
7	0.006	0.015*
8	0.005	0.009*
9	0.119	0.156
10	0.000	0.000*

* Significantly different sets