WAGENINGEN UNIVERSITY
LABORATORY OF ENTOMOLOGY

The spread, abundance and life history of *Harmonia axyridis* in the Netherlands

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Content

Preface

This thesis was done as the last part of my Master Biology at the Wageningen University. The size of this thesis was 30 ECTS. For me, this particular thesis was the perfect opportunity to further diversify the number of scientific topics I had been in contact with and improve my abilities as a scientist. Prior to this thesis, I have completed a thesis in Grenehousegas modelling, a theoretical ecological subject and an internship on Lepidoptera biodiversity at Alterra. My main aim for this research was to work with many different aspects of a single subject and work more closely to an existing project than I have previously done. This also aimed to improve my scientific writing and communication skills.

During my thesis, I have succeeded in working with all three topics of my research title: Spread, abundance and life history. I have done 4 distinct experiments, of which 2 are still ongoing since nature does not allow convenience steer it. I have also subdivided my report into 4 different chapters, each covering an experiment. These chapters can be read individually, though they do connect together. The main aim to gain a better understanding of the position of *Harmonia axyridis* in the Netherlands is central in all four topics.

I hope you enjoy reading this report.

With regards,
Jos Abma BSc
Wageningen
28st of June 2009

Acknowledgements

I would like to thank a number of people for their support during this thesis. First of all Lidwien Raak-van den Berg who has done a truly superb job as my supervisor, providing me with ideas, but also encouraging me to develop my own ideas and working them out. I would also like to thank Peter de Jong who has been a valuable source of information and helped a lot in refining the experiments I worked on during this thesis. I would also like to thank Joop van Lenteren for being my examinator.

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I would like to thank the group of Entomology for providing a wonderfully encouraging and motivating working environment.

Introduction

The displacement of animal species to new habitats is a primary concern for conservation biologists worldwide. Such concerns are no exception in the Netherlands. With the ongoing globalisation of the world's economies, the purposeful release of exotic animals and the effects of climate change, exotic species are here to stay. To protect the native habitats of the Netherlands against exotic species, the effect of exotic species on the native ecosystems must be understood. Such effects are often related to the species' effects on the local populations of competitors, their prey species and possibly predators on the species. An exotic species' spread, abundance and life history can be of vital importance for understanding what traits a species possesses that allow it to flourish (Townsend et al., 2003; Brown et al., 2008).

Harmonia axyridis (Pallas) (Coleoptera: Coccinellidae) also known as the Multicoloured Asian Ladybird has spread in many regions as an exotic species. *Harmonia axyridis* originally occurred only in Eastern Asia. Its native habitat stretches from Siberia to southern China. It is also native to Japan. In its native habitat, it is known as a voracious generalist predator (Brown et al., 2008; Adriaens et al., 2008).. *Harmonia axyridis* preys primarily on a wide range of aphid species, though it will frequently be involved in the predation of other insects. This second group of prey includes the useful predation of several other plague insects. It also includes a number of insects that also live primarily on aphids, the so-called intraguild predators (Koch et al., 2003; Pell et al., 2008; Brown et al., 2008) Despite the effectiveness of *H. axyridis* as an biological pest control species, its traits meant that it had a high probability of invasive behaviour in other habitats (van Lenteren et al. 2003).

Despite these traits, *H. axyridis* was released in north America as early as 1916 for purpose of biological control. The most intensive release of the animals was concentrated in the 1970s and 1980s (Koch & Galvan, 2008). In the Unites States and southern Canada *H. axyridis* has since become established and has displaced many of the native species (Hesler & Kieckhefer, 2008; Mizell 2007; Finlayson et al, 2008). The release of *H. axyridis* in Europe is more recent. It was used in France during the early 1980s and in many other European countries in the late 1990s. In most of these countries, evidence of the species being established has been found (Brown et al., 2008).

In the Netherlands, *H. axyridis* was used between 1996 and 2003 as a biological control agent on aphids. In 2002, the first wild individuals were found. Since 2004, the species was found in increasing numbers (Brown et al., 2008; Loomans, 2004). In the last years, the number of observations and the number of individual adults seen has increased (waarnemig.nl, see fig 1), indicating that *H.axyridis* has established itself as a species in the Netherlands.

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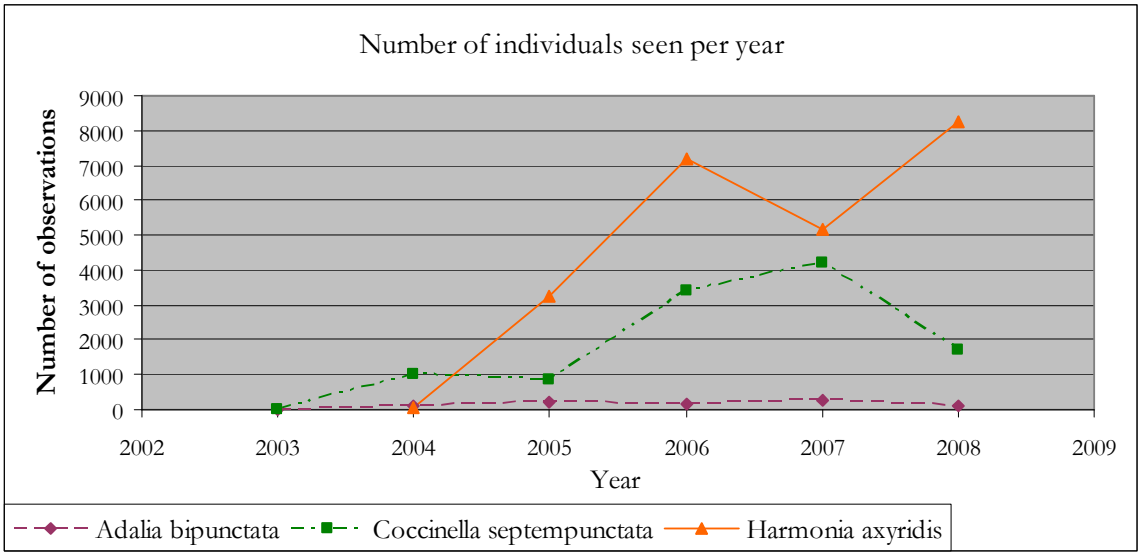

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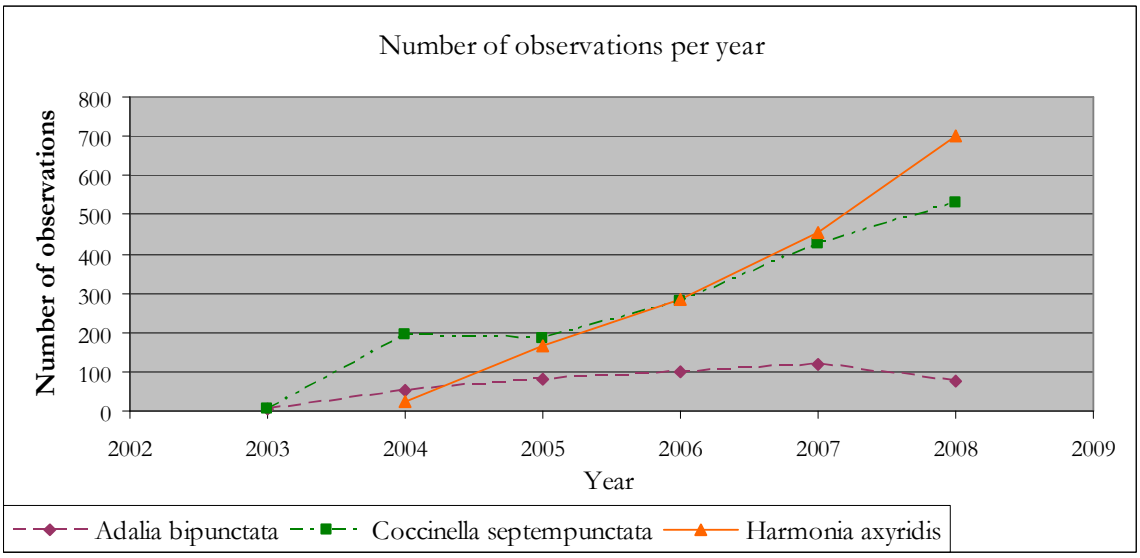


Fig 1 The number of individuals seen and the number of observations per year for *Harmonia axyridis* and reported to waarneming.nl. (**Disclaimer:** Data used in these graphs is only meant for this thesis and not meant for use in commercial gain. Waarneming.nl cannot guarantee the correctness of this data)

Little more is known about the position that *Harmonia axyridis* has gained in native Dutch ecosystems and what impact the species has on the native species of coccinellids. This thesis aims to provide the first framework to improve this understanding. Due to the time limitations of this particular subject, it will not be possible to completely investigate any of the subjects. Instead this research is aimed towards providing the first answers to a growing understanding of the spread, abundance and live history of *H. axyridis* in the Netherlands. A secondary goal is the support of the PhD thesis by ir. Lidwien Raak-van de Berg with spread and abundance data from the nature areas.

1 Phenological exploration

1.1 Introduction

Ecological phenomena are very important in the dynamics of ecological systems. They indicate the biological progress of the seasons and as such provide a wealth of knowledge into the dynamics underlying the interactions of species. In research, such phenological phenomena can aid in pinpointing the best time for certain types of research or field work. Monitoring phenological events can therefore be very important. For most native species, their phenology is more-or-less known, though climate change is currently considered to be a major factor in altering such phenological events (Parmesan 2006). By definition, exotic species are new to their habitat. Their phenological timing is therefore unpredictable, though some conclusions can be drawn from the phenological characteristics they displayed in their native habitats.

H. axyridis is well known as a top intraguild predator in its native habitat and in the U.S.A. (Koch et al., 2008; Mizell, 2007; Finlayson et al., 2007). Both larvae and adults are extremely voracious and this trait makes them a treat not only as a competitor for resources, but also as a potential predator for the aphidophagous insects in the Netherlands (van Lenteren et al., 2008; Pell et al., 2007; Majerus et al., 2006). Currently, *Harmonia* is known to act as intraguild predator towards *Adalia bipunctata*, *Coccinella septempunctata* and a number of other intraguild prey (Yasuda & Ohnuma, 1999; Ware et al., 2008; Ware et al., 2008 (2); Michaud & Jvoti, 2007). Intraguild predation pressure by *H. axyridis* is avoided in its native habitat by temporal divergence of the larval development (Pell et al., 2007). It is also unclear if native species in the Netherlands are temporal different in their oviposition and larval development. It is therefore not unlikely that *H. axyridis* will have a negative effect on the native populations of aphidophagous and soft-bodied insects.

When compared to a native species such as *Adalia bipunctata*, *H. axyridis* starts its reproductive cycle late in the season where it is invasive (Adriaens et al., 2008). Larval development will therefore usually take place after the period with the highest aphid abundance. *H. axyridis* larvae will hence find themselves faced with a lesser aphid abundance. Due to its ability to complete its larval cycle on diets other than aphids (Kalaskar, 2001; Koch et al., 2003 (2); Pell et al., 2007), *H. axyridis* larvae can then continue to develop on a diet of heterospefic (intraguild) prey and through cannibalism.

From surrounding countries, a number of surveys are known. In Belgium, the arrival and spread of *H. axyridis* was exclusively followed and documented. The group concluded that the presence of *Harmonia* is widespread and in great numbers. *H. axyridis'* spread was mainly concentrated in urbanised landscapes.

The species appeared from winter aggregations during the second half of April or the first half of May (Adriaens 2008). In the United Kingdom the early invasion patterns were established for *H. axyridis*. It showed a strong spread of the species through the south of England and into Cornwall and Wales (Brown et al. 2008). A climatological model also showed that it is likely that *H. axyridis* finds little to no hinderance of the climate in its spread (Poutsma et al. 2008).

The main aim of this study was to get a broad idea of the presence of *H. axyridis* in the Netherlands. As a primary goal, an estimate was to be made how wide-spread *H. axyridis* in the Netherlands is.

1.2 Materials and methods

As very little is known about the spread of *H. axyridis* in the Netherlands, preliminary research (personal communication with Lidwien Raak-van den Berg) suggests that *H. axyridis* occurs primarily on trees with many aphids, such as *Tilia* and *Acer*. These trees were given priority during the experiment. At the same time, the Belgian observations point to a much more wide distribution of *H. axyridis* over ecosystems. As such, we were mainly interested in the general spread of the species and the timing of the key life-cycle events.

Seven (7) locations were chosen according to their strongly differing ecological conditions. These were located in a semi-straight line near the city of Wageningen and will be referred to as a 'transsect'. The main differences between the different locations could be summarized in the types of environment (nature and urban), the type of management (nature conservation, agricultural usage and urban gardening) and type of soil (clay and sand). In addition, this set-up allowed us to scout a number of locations for the suitability of these locations for the life history experiment.

Table 1.1 – The locations visited in the phenology exploration.

Location name	Description
Blauwe Kamer	This location is a natural wetland area in a bend of the Rhine river. The subsoil is river clay. Water level in the area is near ground level and the ground water is fed primarily from the Rhine river and sedimental water from the Grebbeberg. In the area, large grazers (horses and cattle) keep the vegetation low. The vegetation consists primarily of wet grasslands with shrubs and trees loosely located through out the landscape. Most of the trees are found on old defensive structures that cause ground level elevation.
Gebbeberg	This ridge in the landscape is an old glacial deposit. As such, it consists primarily of sand. There is no grazer management on its slopes. The primary type of forest on the hill is dedicious, but a number of patches of open ground and pine forest are also present. The forest floor is dominated by brambles. In general there are very low light conditions on the forest floor. The area searched is a south-east facing slope. Due to the amount of sun exposure as a south-facing slope, this indicates that the general area will be slightly warmer than the other natural areas. Wind is also expected to be low here, due to the direction of the slope.
Binneveld (Schiphorst)	In the Binneveld, the location will be the tree cultivation farm of Schiphorst. This is an organic farm where no chemical treatment is applied to the trees. Machinal activity is limited to mowing the undergrowth once a year. The subsoil is sand on

	clay with a half-high groundwater level. The undergrowth is a sown in mixture of herbaceous plants. Most of the trees are young (1-5 years old) and spaced out regularly. (During the month May, Schiphorst was observed as part of the Life History experiment. Data is located in the part of the report)
Vineyard Wageningse Berg	This organic vineyard is located at the Wageningse Berg, a location similar to the Grebbeberg in origins and subsoil. The field is facing to the North-West, but lies in a sheltered area between trees. Manure is applied once a year and there are regular sprayings with non-toxic organic pesticides. The vines are located in rows with about 2 meters separating between rows. The sides of the field are covered with brambles and a number of herbaceous plants.
Small arboretum	This is a rose-garden maintained by the Wageningen University. This location is especially interested, because a number of Asian *Rosaceae* species are located in the garden. These plants are planted in a random spread-out fashion. The subsoil is mostly eutrophic sand with a high groundwater level. The *Rosa* plants investigated were mostly woody shrubs.
Large arboretum	In the larger arboretum a broader selection of plants was available, but the focus of the study was on the *Tilia* trees located along the walking paths. These are big, mature trees on a sandy subsoil.
Rosa nigosa bushes	These bushes were located next to the Ritsema Bosweg and are planted by the municipality. They are mature cultivar plants that are a number of years old and grow in dense packs. The subsoil for these plants is uncertain, as they are both planted and grow near a brick road. A brick road is always founded upon a mixture of sand. In addition, traffic will lead to deposits in the bushes.

Fig 1.1 The locations on the transect near Wageningen.

In order to minimize disturbance at the locations that were visited, searches were done visually. Per location, a standard walking path was plotted that could be walked in a relatively short time (+/- 10

minutes). Walking in such a way ensured that multiple locations could be visited in a single day, while still allowing for a relatively broad area to be searched. This path was then walked every week and plants surrounding the path were checked for the presence of ladybirds (adults, larvae, pupae and eggs). In this search, all species of ladybirds were included, not singularly *H. axyridis*. By recording these finds, it was hoped that an indication could be found if native species are present on the same moment in time and at the same location as *H. axyridis*. The trees and shrubs were checked visually and a number of leaves were turned to view their lower side. A general impression of the phenological development and the weather were also recorded to support the finds.

1.3 Results and Discussion

The finds of insects were recorded on a weekly basis from March 2nd through to the 16th of June. Measurements are still ongoing as of the writing of this report.

During the month of March, no insects were found on any of the plants. After that, regular observations were made (see table I.1 in Appendix I). The most common species found was *H. axyridis*. Frequent observations were also made of *C. septempunctata* and *A. bipunctata*. Almost all of these observations were made of adult females, generally resting or walking. Feeding or ovipositioning behaviour was not recorded during the measurements on any of the seven locations. In addition, females were rarely found twice at the same location. Eggs and larvae were found on the *Rosa nigosa* bushes, the large arboretum and the Schiphorst location. Pupae were found on the Schiphorst location as well.

The finds of larvae are therefore primarily in semi-natural and urban areas. This is likely related to the abundance of aphids on these locations. The *Rosa* bushes had a low density of aphids. So did the Schiphorst location and the large Arboretum. Larvae found on the large Arboretum were only found towards the end of the aphid abundance (see Chapter 2). These locations still had a presence of aphids, while the more natural areas of the Grebbeberg and Blauwe Kamer had no aphids at all.

This distribution of observations is interesting. In other countries, such as Belgium, the spread of *H. axyridis* is relatively uniform, but also showed a trend towards urban areas for the primary larval development (Adriaens et al., 2008). Aphid abundance could play an important role in this spread, since no other coccinellid species were observed in the natural areas. This would point to an unsuitability of some of the investigated areas for the presence of larval coccinellids as a whole due to the lack of an abundant aphid population.

The distribution of aphids and coccinellids could be related to the health of the vegetation. Vegetation monocultures are known to be very susceptible to pests. The susceptibility is caused by a lack of distance

between plants and a reduced fitness effect of monoculture vegetation due to niche-centred competition for resources. While in natural systems, niche differentiation allows for better exploitation of natural resources and does not have a fitness effect on the vegetation, making this vegetation more able to resist herbivores such as aphids. Also, the more heterogenic distribution of the vegetation makes it harder for aphids and other pests to spread between plants (Townsend et al., 2003). As a result, these ecosystems would provide less vegetation with abundant aphid populations and would become less suited for the coccinellids.

For the achievements of the primary goals of the transect, the method chosen was most likely of sufficient quality. The choice of locations and vegetation sampled could have done a bit better. *Tilia* trees were sampled only in the most nature specific areas and in the Arboreta, while the biggest developing aphid and *H. axyridis* populations were primarily found on the trees located near paved structures. The *Rosa nigosa* bushes that were measured did not have a concentration of larvae. It would therefore likely have been better to observe the *Tilia* trees.

1.4 Concluding remarks

During this experiment, very little attention has been given to the role that the herbaceous layer could have on the sheltering of coccinellid species. *H. axyridis* can be found in large numbers in city-lined trees (see Chapter 2). This experiment did not assess the possibility of a more herbaceous-centred existence for *H. axyridis* in the Netherlands. It would be an interesting and potentially important study to include when this survey is repeated next year. Adriaens (2008) found *H. axyridis* primarily on *Urtica* (Urticaceae) and Acer (Sapindaceae), which supports the suggestion that a focus on the herbaceous layer.

The most important obstacle to the current survey is the scale. Though it is able to function as an indication, true figures are still unknown. A good way to improve this knowledge would be a project in which the general public can be asked to be on the lookout for these insects. An example would be the Natuurkalender (Naturecalendar), which gathers data from thousands of volunteers throughout the Netherlands on the phenological characteristics of species. If *H. axyridis* could be included in this survey, it would provide accurate data at the appearance date of the species.

2 Life-history experiment

2.1 Introduction

2.1.1 Life history

The life history of a species is determined by many factors, both biological and environmental. Different habitats have different biological and environmental requirements. This means that in different habitats, species will have different life histories. Species new to an ecosystem are not yet adapted to the conditions present in that system. The life history traits of such a species will therefore not be optimal. Non-optimised life history traits can lead to a reduction in fitness. This applied both for individual reproductive success and population stability (Parmesan, 2006).

An adaptive life history is therefore important in the survival and establishment of exotic species in new habitats. The life history traits of a species can both be fixed and flexible. Fixed life history traits are traits that remain unaltered, regardless of environmental and biological effects. Flexible life history traits are the traits that will be different depending on the conditions in which the organism lives. Ectothermic organisms, like insects, are especially restricted in their life history due to their dependence on temperature for their ability to function. This also means that some of the life history traits (e.g. the development rate of larvae) depends directly on the temperature.

Harmonia axyridis is an Asian ladybird that has become invasive in the Netherlands since 2002, when it was first found. Though very little is known about the current spread of the species in the Netherlands, it is considered to be an established species (Loomans et al., 2004). Since 2004, it has been seen in greater numbers and is expanding its range over the whole of the Netherlands (Loomans et al., 2004; waarneming.nl, 2009).

In its native habitat, *H. axyridis* is a dominant generalist aphidophagous predator (Koch et al., 2003). It is also the dominant intraguild predator within the aphidophagous guild. *H. axyridis* females that emerge from their winter dormancy have been found to lay their eggs during the highest aphid abundance of the season. This allows a rapid larval development during the first stages. When the later larval instars are reached, however, the larvae are more likely to find themselves with a low abundance of aphids (Yasuda & Shinya, 1997). Due to this, intraguild predation and cannibalism has become an important factor in the life history of *H. axyridis* (Osawa, 1992; Osawa 1993). Larval mortality of *H. axyridis* in its native habitat is especially high in the 4th instar larvae (Yasuda, 1997; 93.5% according to Osawa (1992)). This is attributed to the low densities of available aphids for the larvae. In other larval stages, mortality is lower (± 50%). This can likely be attributed to the better availability of food and the faster rate of development through

these larval stages. In it's forth instar, *H. axyridis* will likely be able to succeed in its typical behaviour of a cannibalist as a means to survive the lack of aphids.

As *H. axyridis* is an exotic species in the Netherlands, less is known about the interactions between *H. axyridis* and native species, including its position as an intraguild predator. This is due to the lack of co-evolution between *H. axyridis* and native coccinellids. It is known that *H. axyridis* is a dominant intraguild predator on species such as *A. bipunctata* and *C. septempunctata* (Yasuda & Shinya, 1997; Yasuda & Ohnuma, 1999; Pell et al., 2007; Michaud & Jvoti, 2007). It is known that *H. axyridis* is resistant to both cold and warm conditions. The temperature is therefore unlikely to be a limiting factor for the success of *H. axyridis* as an invasive species (Koch et al., 2004).

2.1.2 Life cycle of H. axyridis

Hamornia axyridis, as a Coleoptera (beetle), has a holometabolic life cycle, undergoing a complete change between larval and adult stages. Adult females lay eggs in batches between 10 and 40 eggs (Stathas et al., 2001). After a number of days, depending on the environmental conditions, larvae will hatch from these eggs. These larvae will feed primarily on eggs, eggshells and each other for the beginning of their life, but also frequently start predating on aphids. The larvae will then go through a number of moulting phases, creating four to five distinct larval instars. During the latter instars, the larvae will feed primarily on aphids, as long as these are available. If aphids are scarce, other sources of food can be exploited by *H. axyridis* larvae. These included other insects of similar size and some types of fruit. Larvae will then attach themselves to a single location and pupate. After a number of days, adults will emerge from these pupae. The development time of these larvae will be dependent on a number of environmental conditions and biological conditions such as the availability of food.

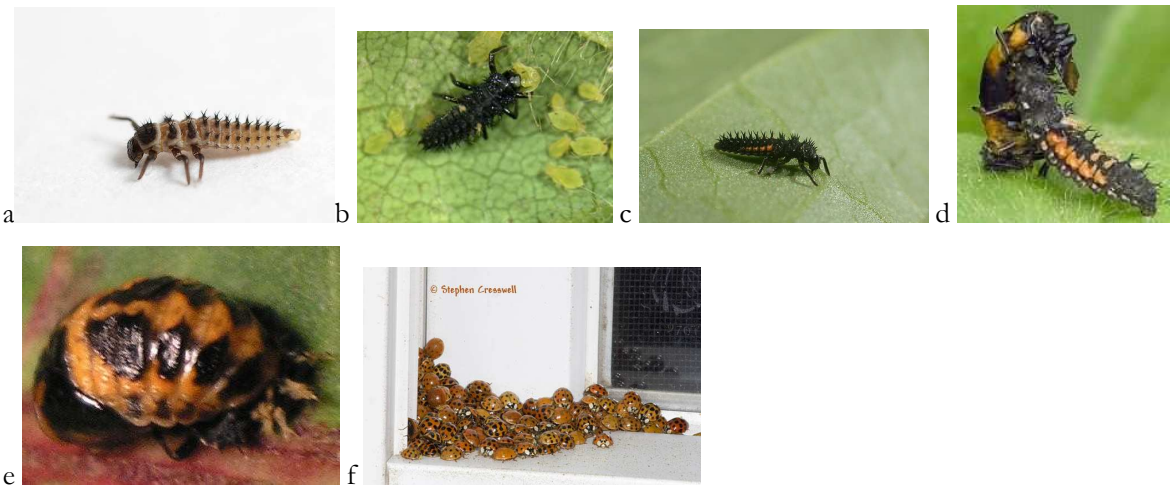


Fig 2.1 The different life stages of *H. axyridis*. a: 1st instar (L1), b: 2nd instar (L2) eating aphids, c: 3rd intar (L3), d: 4th instar (L4), e: pupa (P) and f: adults in winter aggregations (A).

2.1.3 Life history experiment

In this life history experiment, the main focus will be concentrated at the time-line of development. The causal environmental relationships underlying the developmental characteristics of the *H. axyridis* will not be as extensively studied. A measure of the aphid densities will be considered as the only biological factor. Other species of coccinnelids will be considered as an indication of the relative developmental timing of *H. axyridis* compared to native Dutch species.

2.2 Material and methods

In order to establish a regular pattern of daily measurements, a method was to be found that allowed for accurate daily measurements, while also taking sufficiently little time to be done on a daily basis. The ease of the observations was also important, since I do not own a car to allow for the transport of much research material (such as a ladder). Lastly, the research method had to be non-destructive and non-intrusive. This would ensure that the population suffered no effect from the monitoring. Because it matched these requirements, the method for this search was derived from Yasuda and Shinya (1997).

The locations of sampling were picked according to the occurrence of ladybird eggs or larvae - indiscriminent of species- if also aphids were found on the same location. On each of these locations, three trees were selected that were not physically connected to one-another by branches. In two locations, these were of the genus *Tilia* (locations called 'Haarweg' and 'Marijkeweg'), on the last the trees were of the genus *Acer* (location 'Schiphorst') (see Fig. 1.1 in chapter 1). The size of these trees was significantly different depending on location; Schiphorst trees were all young trees that were less than 2m in height and could be surveyed in full. The Haarweg trees were all young, but grown trees, modelled with horizontal branches. Of these trees, only the lowest branches were sampled. Sampling the lowest branch The Marijkeweg trees were all older trees of significant height (+/- 10 meters heigh) and foliage. Only a single (large) branch was picked from these trees for sampling. All these branches were picked to be disconnected from other branches, if possible, and have roughly the same amount of foliage as the Haarweg lower branches.

Branches were sampled daily between 0800 and 1900 hours. This ensured that daylight was present, the temperature was within day-specific range and no measurement error was made based on light conditions. Sampling was done visually of all branch and leaf surface. Contact with the branches was kept to a minimum and only used if pure visual survey was impossible (e.g. strong wind preventing leaves from being surveyed on both sides). All surveys were done from underneath the branches to improve efficiency (the eggs and larvae of ladybirds have a characteristic silhouette when viewed on a leaf against daylight conditions). Both sides of a leaf were examined whenever possible. The numbers, species and stages of these ladybirds larvae were recorded. On each location, the number of aphids was counted regularly. This was done by counting the number of aphids on 10 separate random leaves. The stage of development of the aphids was not noted. In addition, special circumstances (such as heavy rain, fallen branches, etc) were

also recorded to provide clues for sudden shifts in larval numbers. Sampling was continued until no more juvenile larvae were present on the tree, at which time the tree was no longer monitored for purpose of the life cycle experiment. Monitoring continued as part of the phenology experiment to observe whether new generations would occur.

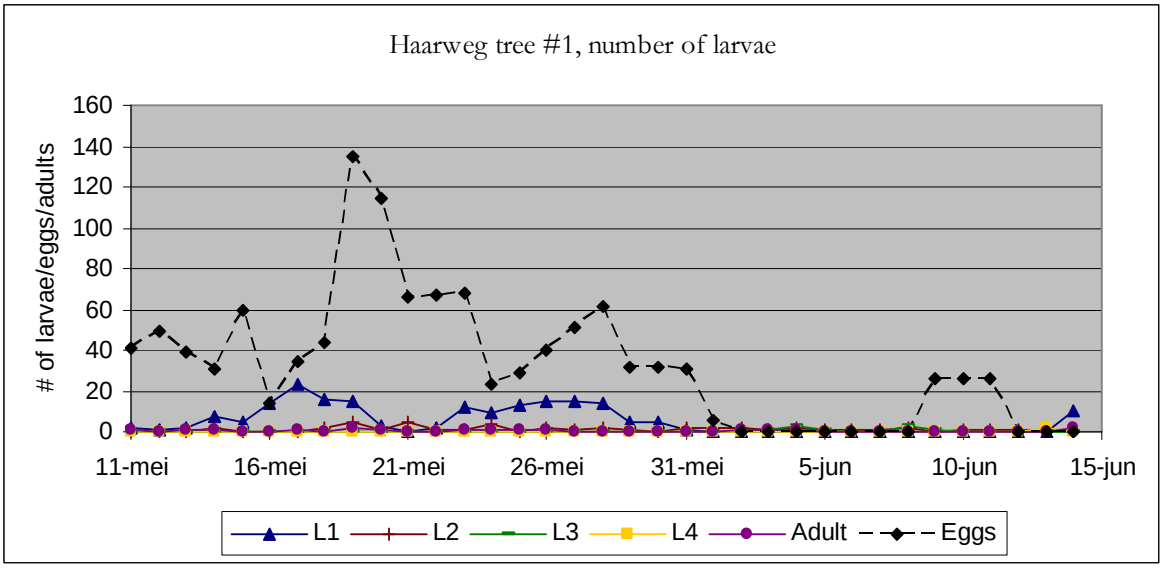
Development time for the various larval instars was estimated using strong abundance shifts as an indication. These shifts were characterised in strong decrease of a younger larval instar that were observed at the same moment of a strong increase in the older instar. The average between the observed trees was then used as an average measure of developmental time. This method was used since individuals were not tracked and could therefore not be directly linked to their moulting phases.

Once during the experiment, three other and smaller branches were sampled on the Marijkeweg location. These branches were then cut and stored in sealed container bags. These bags were frozen at -4°C for a number of days to ensure the death of all larvae. The branches were then sampled again in a tray to ensure a 100% observation of all animals. Original sampling was compared to the 100% sampling. This was then used as an indication of sampling efficiency.

2.3 Results

2.3.1 Haarweg location

None of the the Haarweg location trees showed a continuous population of *H. axyridis* larvae (see fig. 2.2). During the measurement period, new egg batches were found regularly. This can be seen in the strongly fluctuating number of eggs that was found. None of the 1st instar larvae that hatched from the batches were continuously found on the trees through the other larval instars. The species of adults found were *Harmonia axyridis*, *Adalia bipunctata*, *Coccinella septempunctata*, *Propylea quatuordecimpunctata* and *Calvia quatuordecimguttata.*



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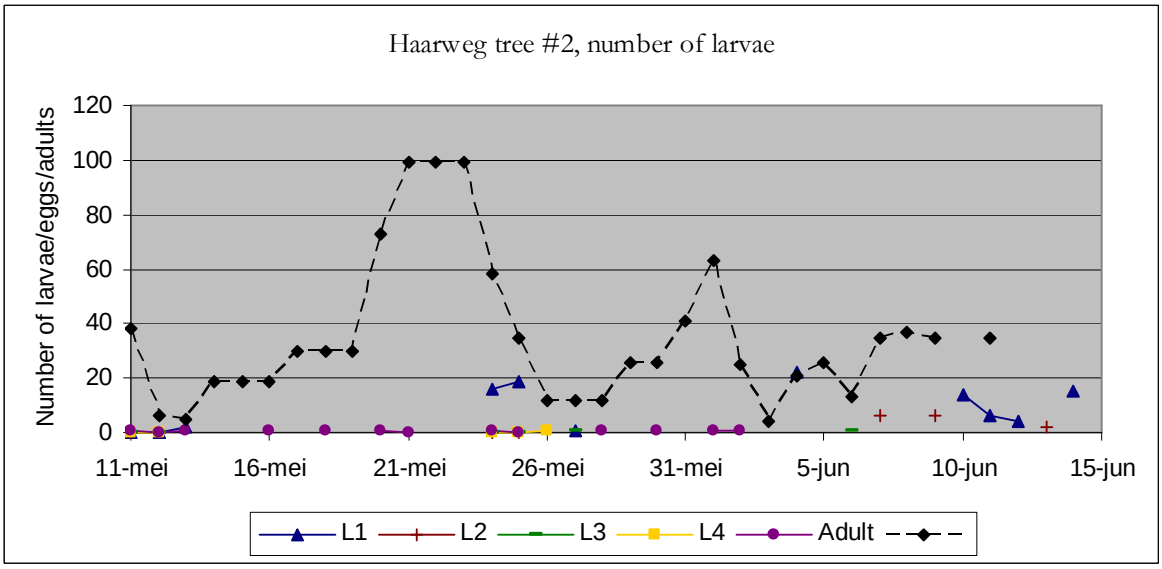

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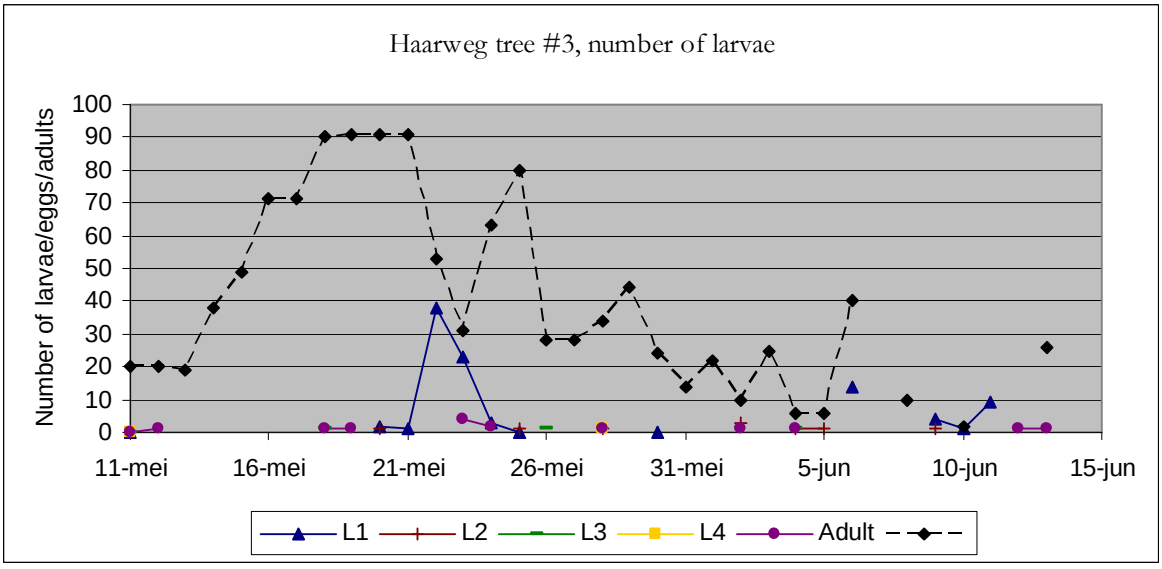

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Fig 2.2 The number of larvae/eggs/adults found on the Haarweg location of the 1st tree sampled.

2.3.2 Marijkeweg location

Measurements on the Marijkeweg were done from the 11th of May to the 16th of June. On the Marijkeweg location, both 1st and 2nd instar larvae were found at the beginning of the measurement period (11th of May). On all trees, continuous larvae populations were found. These populations were studied through all larval instars. Moulting larvae for all instars were found. Hatching larvae were also observed. First instar larvae were observed between the 11th and 17th of May. Second instar larvae were observed between the 11th and 24th of May. Third instar larvae were observed between the 11th and 28th May. The largest number of third instar larvae were observed on the 17th of May. Fourth instar larvae were observed from the 13th of May until the end of the measurement period. The largest number of fourth instar larvae were observed on the 4th of June. A single observation of predation by a non-coccinellid predator of a 1st instar larvae was done during the measurement period. Three observations of cannibalism were made.

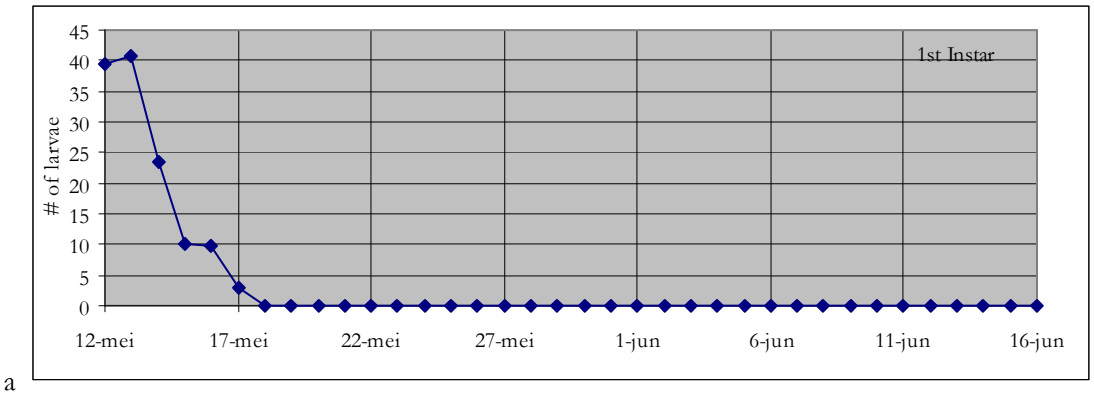
Shifts of larval populations were used to estimate the average development time. A summary of this can be found in table 2.1. The average time was used as it was assumed that the variation between the tree Marijkeweg trees is mostly random.

Table 2.1 The development time per instar, categorised by Marijkeweg tree

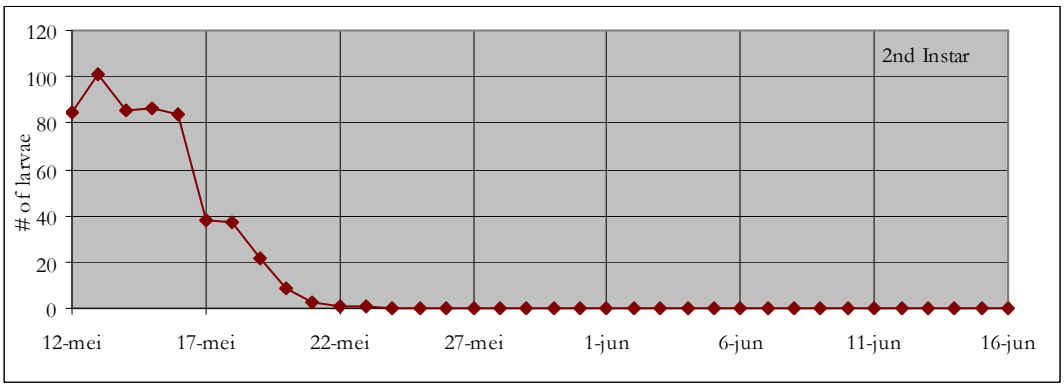
	Larval instar			
Tree	*First – Second*	*Second – Third*	*Third – Fourth*	*Fourth - Pupa*
#1	2 days	1 day	6 days	24 days
#2	2 days	3 days	8 days	23 days
#3	2 days	4 days	8 days	25 days
Average time	2 days	2.7 days	7.3 days	24 days

The larval populations through time were plotted in both Fig. 2.4 and Fig 2.5. In Fig 2.4 the average number of larvae found on the Marijkeweg trees was plotted, to give an impression of the average larval population progression. Figure 2.5 gives the larval progression through time for each individual tree. This could be used to support the average development time for the larval instars and give an impression of the variation between the trees on the Marijkeweg.

At the end of the measurement period (16th of June), the larvae were all in their fourth instar and fairly large in size. After the measurement period was ended, most larvae pupated within days.



a



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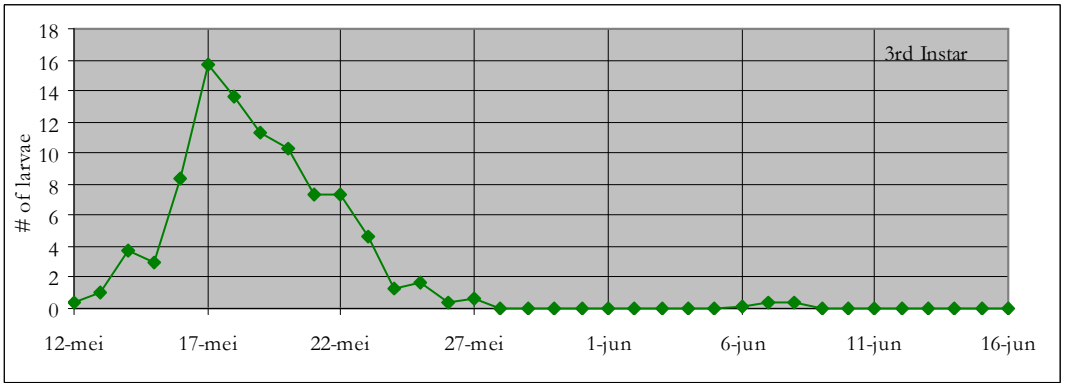

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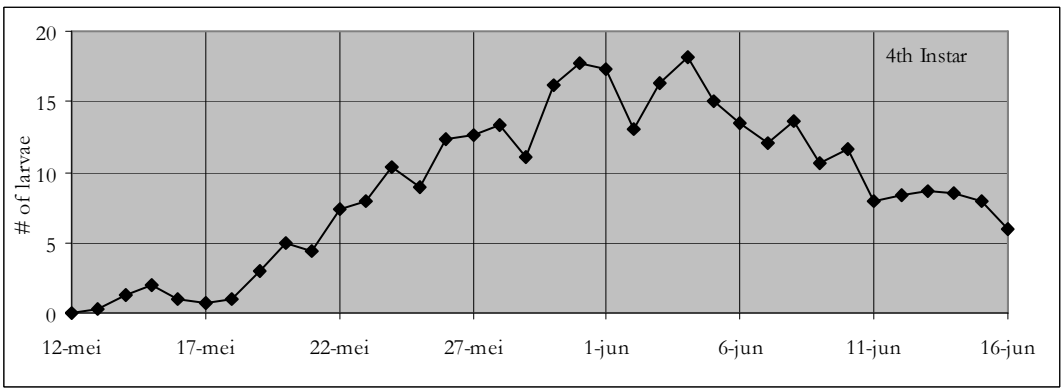
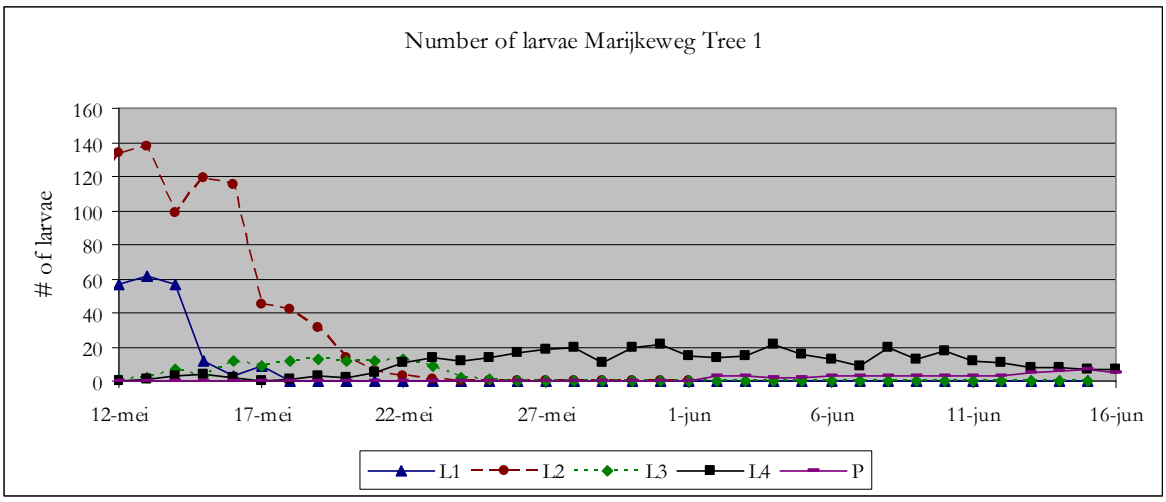


d

Fig 2.3 The average number of larvae found per larval stage for all three trees on the Marijkeweg location. Measurements were started at the 12th of May and ended at the 16th of June.



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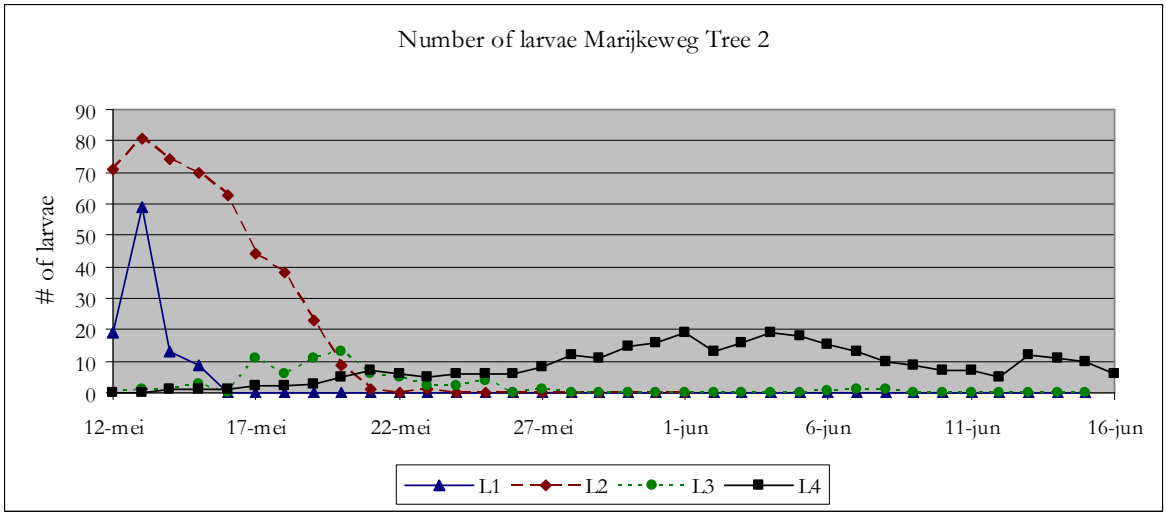

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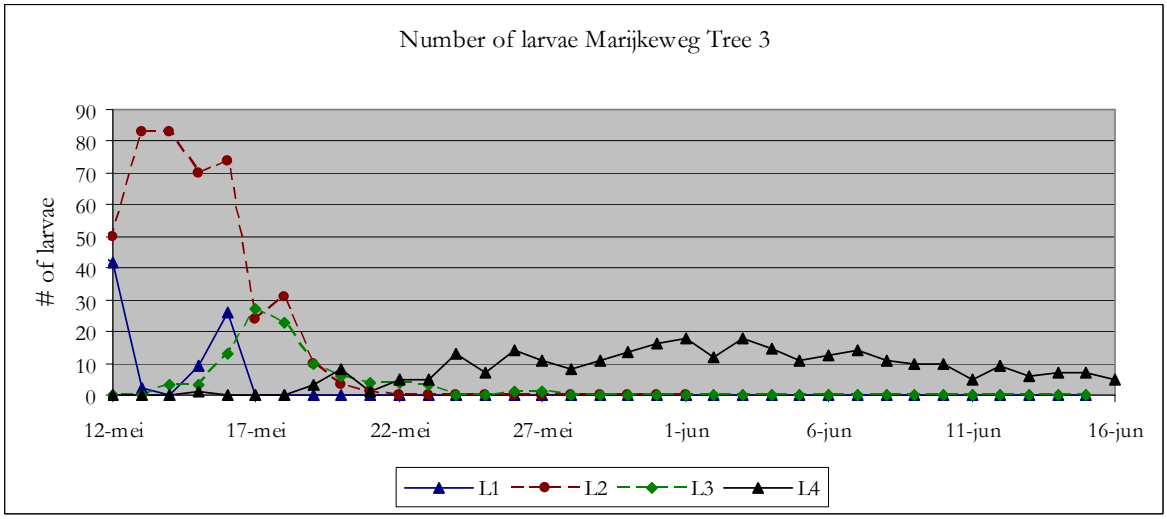


c

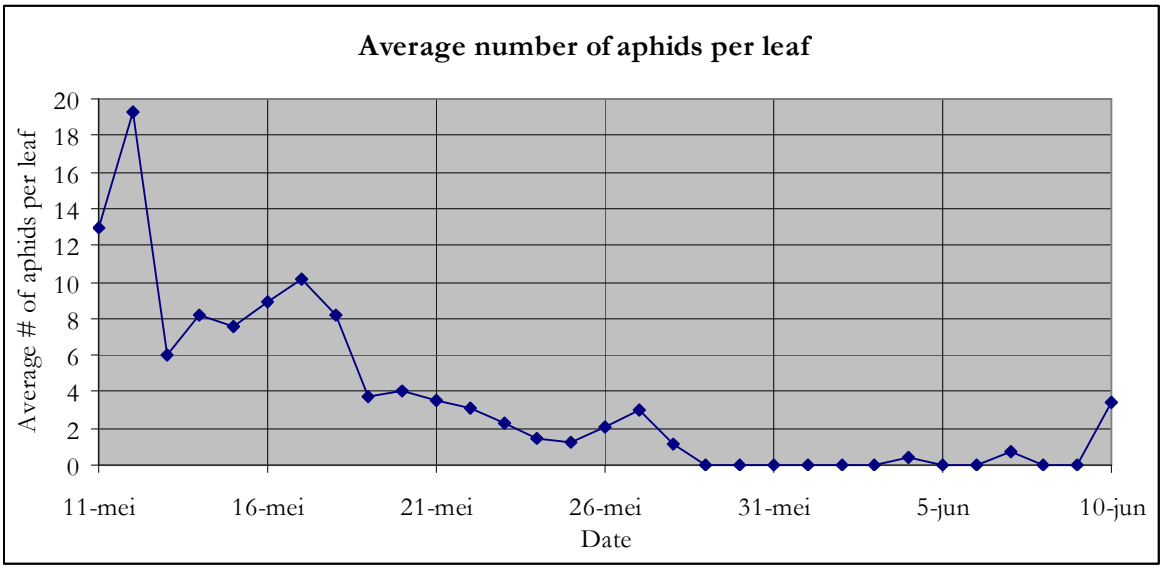
Fig 2.4 The number of larvae found on one of the Marijkeweg trees for all larval instars and the pupae. Measurements were taken from the 12th of May until the 16th of June.

2.3.3 Schiphorst location

On the Schiphorst location, insects eggs were observed on all days from the 11th of May to the 22nd of May. Upon hatching, no coccinellid larvae were found. The eggs observed were instead *Lepidoptera* eggs and as such useless for the research. Daily measurements were therefore stopped at the Schiphorst location after the 22nd of May. A single young instar (L1/L2) *H. axyridis* larvae was observed on one of the Schiphorst trees. This larva was found on the 8th of June to be in its pre-pupal stage. On the 15th of June it was found to be in its pupal stage.

2.3.4 Aphid population

The aphid population found on the trees, though randomly counted, varied greatly by measurement day and place. The larger, infested leaves had many (up to 90) aphids on them (see Fig 2.5b). Most of these aphids were juvenile (small and wingless), with a maximum of 2 adult aphids (winged). During the measurement period, the average number of aphids per leaf dropped to less than 1 (see Fig 2.5a). At the end of the measurement period, the majority of leaves was without aphids. Those leaves that still had aphids were generally limited to 3 or less aphids, all of them adults (winged). This indicate a strong decrease in aphid abundance over the measurement period.



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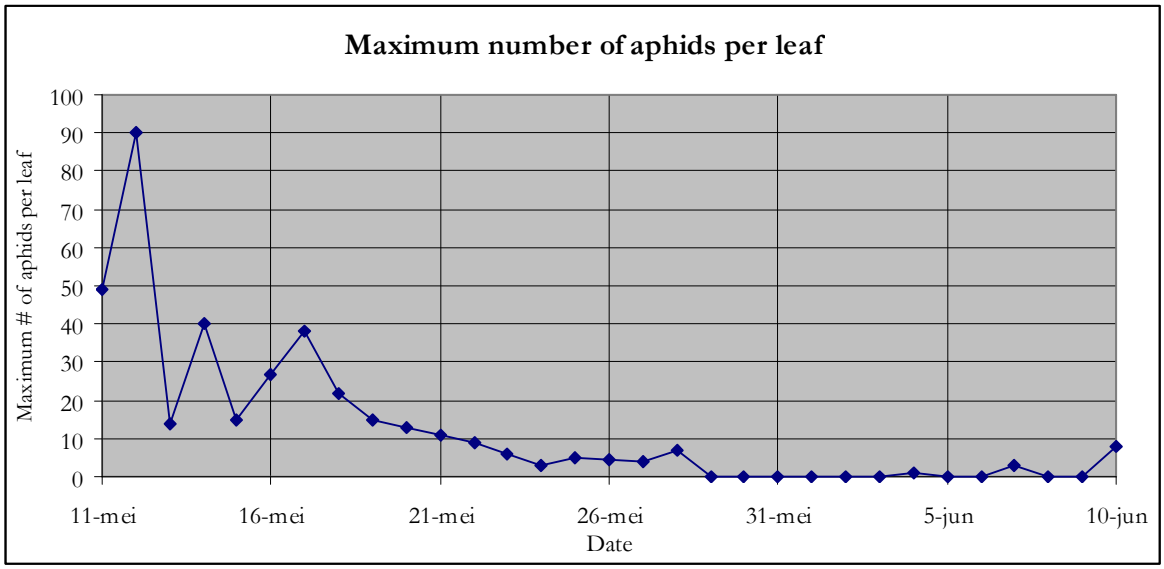


b

Fig 2.5 The average and maximum number of aphids found per leaf on both the Haarweg and Marijkeweg locations combined.

2.3.5 Branch sampling

The branches that were collected to establish the observation efficiency were examined after freeze-killing all larvae. The larvae observed in the field on the branches were all L4 larvae of *H.axyridis* and a single female adult. No pupae were observed. After killing, 100% of the observed individuals were recounted.

2.3.6 Other observations

Observations of other coccinellids were done throughout the period. In most cases these were adult beetles of the species *Adalia bipunctata*, *Coccinella septempunctata*, *Propylea quatuordecimpunctata* and *Calvia quatuordecimguttata*. L3 and L4 larvae of *C. septempunctata* were observed between the 11th and 24th of May on the Marijkeweg. Pupae of this species were not observed, but on the 12th and 15th of June, young adults of this species were observed on the Marijkeweg. These young adults were identified due to their incomplete colouration (slightly yellow-orange rather than bright orange-red).

2.4 Discussion

This discussion will focus primarily on the articles by Yasuda & Katushiro (1997) and Osawa (1992). This was done because the best comparison can be made with these articles, which gives similar population graphs as were created for this experiment, followed a similar protocol, a similar location and used roughly the same number of trees (3 in this experiment versus 3 and 5 in Yasuda & Katushiro (1997)). The main differences are the number of locations (3 in this experiment versus 1 in Yasuda's experiment), the species of trees (*Tilia* versus *Hibiscys*), the time period during which the trees were observed and the size of the trees. He furthermore did the experiment on the Yamagata University terrain as opposed to the Netherlands.

2.4.1 Oviposition

Both Yasuda & Shinya (1997) and Osawa (1992) measured from the first moments eggs were laid and followed the laying of eggs through the season. On the Marijkeweg location, the majority of the larval population was already present when measurements began. Our egg measurements from this location are therefore not accurate. A good comparison with the egg observations by Yasuda & Shinya (1997) and Osawa (1992) is therefore difficult. This comparison could be made on the Haarweg location, where new egg batches were found regularly (see Fig. 2.2). Such a pattern of eggs being laid over a longer period of time is comparable to the eggs found by Yasuda & Shinya (1997), who observed eggs from early may until mid June (see Fig 2.6 and 2.7). On the Marijkeweg no new egg batches were found after the initial population had established itself. This could be related to the presence of absence of larval tracks (see Chapter 4 of this report). Especially third and fourth instar larvae are known to produce these tracks (Doumbia et al., 1998) and the disposition of eggs seems to be reduced by the presence of third instar larvae in both Yasuda's experiment as well as this experiment. This is further supported by the continued egg oviposition in the Haarweg were few to none third and fourth instar larvae were found, whereas Yasuda's observations of new egg batches stop after the appearance of third and fourth instar larvae. It could similarly be argued that the females are at the end of their oviposition period, but this would not account for the absence of continued oviposition at the Marijkeweg location. Due to the lack of accurate egg observations, the mortality of the eggs can not be established. The Haarweg observations, seem to indicate that this mortality is likely high (see fig. 2.2).

2.4.2 First and Second Instar larvae

The observations made of 1st and 2nd instar larvae are closely related to the number of eggs that were found in the trees prior to the first larval hatching. This population development was not completely recorded in the experiment. Directly relating the measurements from this experiment to the data found by both Yasuda & Shinya (1997) and Osawa (1992) is therefore difficult. In our experiment, both these populations were on the decline due to mortality and instar advancement. The start of these populations is not instantaneously, as is shown by Yasuda & Shinya (1997) (see fig. 2.6 and 2.7). It would be logical to assume that a part of the 1st instar population had already entered into the 2nd instar by the time measurements started.

As the number of eggs prior to the 1st instar population and the original 1st instar population build-up are not part of this experiment. Estimating the mortality of eggs and 1st instar larvae is therefore difficult. The comparison is even harder due to the likelihood of a measurement error occurring when comparing big 1st and small 2nd instar larvae. Due to the minimal disturbance method, with purely visual observation, the observations could not be confirmed by sampling the population and viewing the larvae under the microscope.

The residence time and mortality of 2nd instar larvae is also harder to estimate. Oviposition at the Marijkeweg seems to have been concentrated. This has caused the 2nd instar larvae to outnumber the 1st instar larvae. As this experiment did not track the individual larvae as with Yasuda and Shinya (1997), it becomes a lot harder to estimate that residence time as well.

2.4.3 Third instar larvae

Development of the third instar population was observed as a whole. The duration in which they were observed on the Marijkeweg trees were 15, 15 and 9 days for the different trees. This is similar to the time in which Yasuda and Shinya (1997) observed this instar. The big difference is the moment. Yasuda and Shinya (1997) observed the presence of third instar larvae during the middle and end of June, whereas the observations at the Marijkeweg were done in middle to end May, a month earlier. Another striking difference is the numbers in which 3rd isntar larvae were found. Yasuda and Shinya (1997) found these 3rd instar larvae in similar numbers to the 1st instar larvae, while on the Marijkeweg the larval numbers did not come close to this number (max 27 v.s. max 80). Due to the longer residence time in 3rd instar larvae, this would indicate a high mortality.

2.4.4 Fourth instar larvae

The period during which L4 larvae were found was remarkable similar in both Yasuda and Shinya (1997) and the Marijkeweg location. On the Marijkeweg the first 4th instar larvae were found in mid May and continued to be observed until the end of observations considered for this report. Yasuda found L4 larvae in a similar period. The numbers of these larvae found, however, were again different. Where Yasuda and Shinya (1997) found large numbers of these larvae, similar in number to their original 1st instar stage (80 v.s. 50), These numbers were different for the Marijkeweg observations (80 v.s. 20). The continued oviposition found in Yasuda and Shinya (1997), was not present at the Marijkeweg location. Due to the longer residence time of larvae in their 4th instar, a continued oviposition would lead to an accumulation of larvae in the 4th instar population. The absence of this accumulation therefore indicates that the effect of prior oviposition to the larval populations is minimal compared to the last oviposition concentration.

Pupae were observed during the Marijkeweg observations, but due to the timing of this report cannot yet be discussed.

2.4.5 Mortality factors

Yasuda and Shinya (1997)is able to give accurate details about the whereabouts and fate of larvae, while in the experiment conducted here, this was almost impossible to establish. In many cases, larvae simply seemed to have vanished. This was especially remarkable at the 16th of May, when observations of larvae

dropped. Heavy rain had fallen during much of the night and the mortality could be the result of this event. Lab experiments done do show a range of predation characteristics of *H. axyridis* that could be observed (see Chapter 3). The disappearance of larvae could therefore also point towards cannibalism. Another consideration is the relative weight of larval husks. Predated larvae are very light and can easily be moved by wind or rain. If this experiment was to be repeated, it would a good idea to include a method for identifying the causes of mortality in these larvae.

2.4.6 Development time

The developmental time of the *H. axyridis* larvae was considerably longer than the developmental time in the lab. This is of course logical due to the constant, warm conditions in the climate chambers used for those ladybirds,. In addition, these lab-held ladybirds were fed regularly, allowing for a far faster developmental time (10.2 days from hatching (Koch et al., 2003)).

2.4.7 Other observations

The observations of other species on the measured locations were characteristically lacking. The presence of 3rd and 4th instar larvae of *C.septempuncata* during the primary 1st instar larval population could indicate that this species undergoes its development earlier in the season. This way, this species benefit from high aphid densities during their entire larval development. This aphid abundance was clearly lacking during the later larval instars of *H. axyridis*. It does indicate that there is a clear difference between the timing of the larval stages of these coccinellid species. As the size of the larvae is important for their effectiveness in intraguild predation, this temporal divergence could impact upon possible intraguild predation occurring between these species. Smaller *H. axyridis* larvae have lesser defences against predation and are themselves less capable of predation. The earlier development of the *C. septempunctata* larvae would therefore reduce the pool of potential intraguild prey of *H. axyridis* during its later larval instars. At the same time, it could prove less beneficial for the *C. septempunctata* populations if *H. axyridsi* comes to prey on the pupae, which are immobile.

The lack of continuous larval observations at the Haarweg is interesting. The observations would likely indicate that there is no continuous population present at the Haarweg trees. Larvae were observed with intervals, but the lack of continued observation gives the strong impression that all these larvae were subject to mortality during their first and second instars. The abundance on the Haarweg of aphids was lower than at the Marijkeweg, but aphids were present on the trees during the whole of the experiment. The experiment does not give any indication what this cause of mortality might be. A better timing of the start of the experiment would likely allow a direct comparison between Marijkeweg and Haarweg oviposition. Adaptation of the experiment to allow to individual tracking of larvae would better allow for the mortality to be mapped. This could then lead to an understanding of the failure of the Haarweg populations.

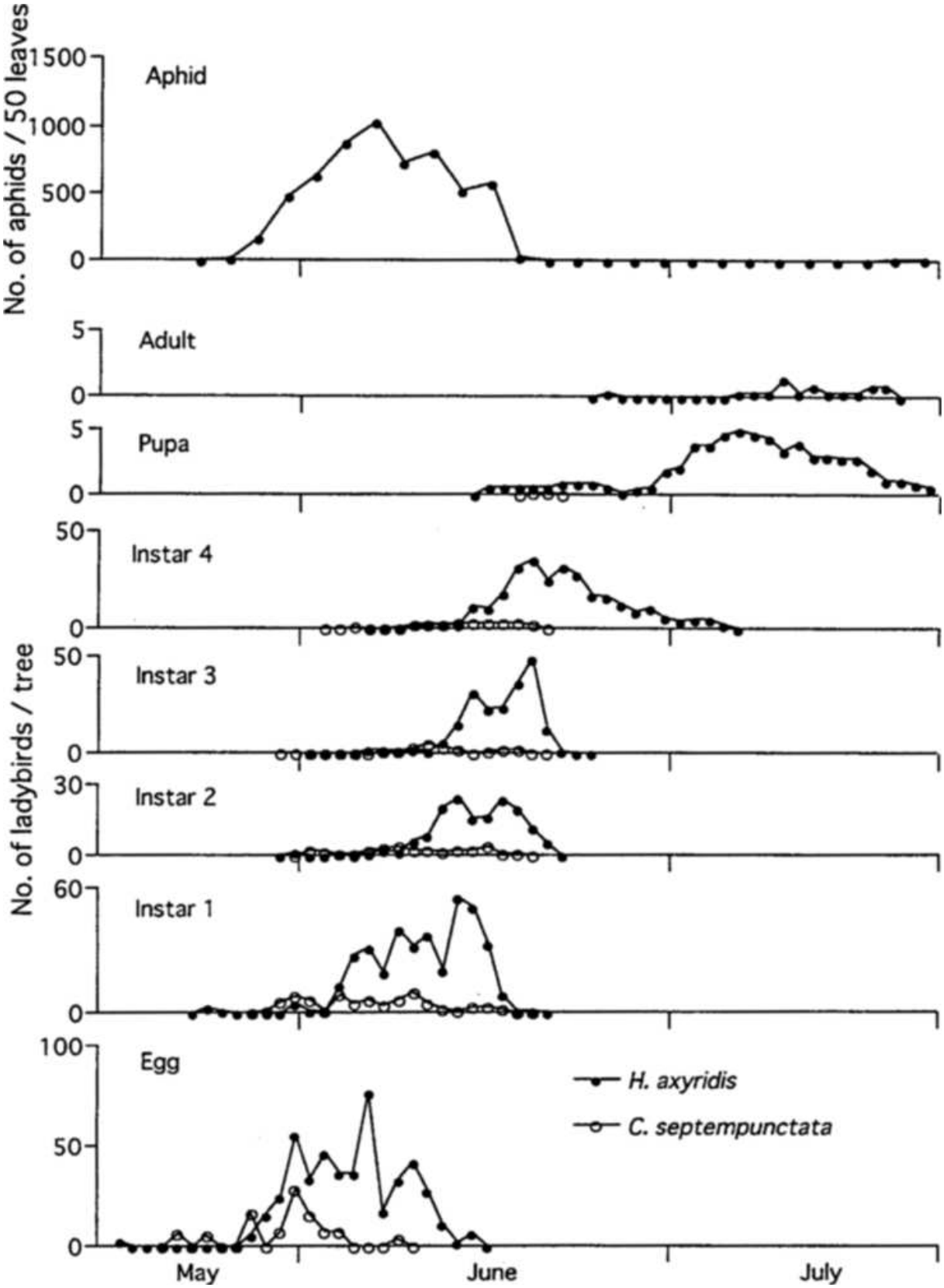


Fig. 2.6 Seasonal changes in the number of aphids and two species of ladybirds in 1993 from Yasuda & Shinya (1997).

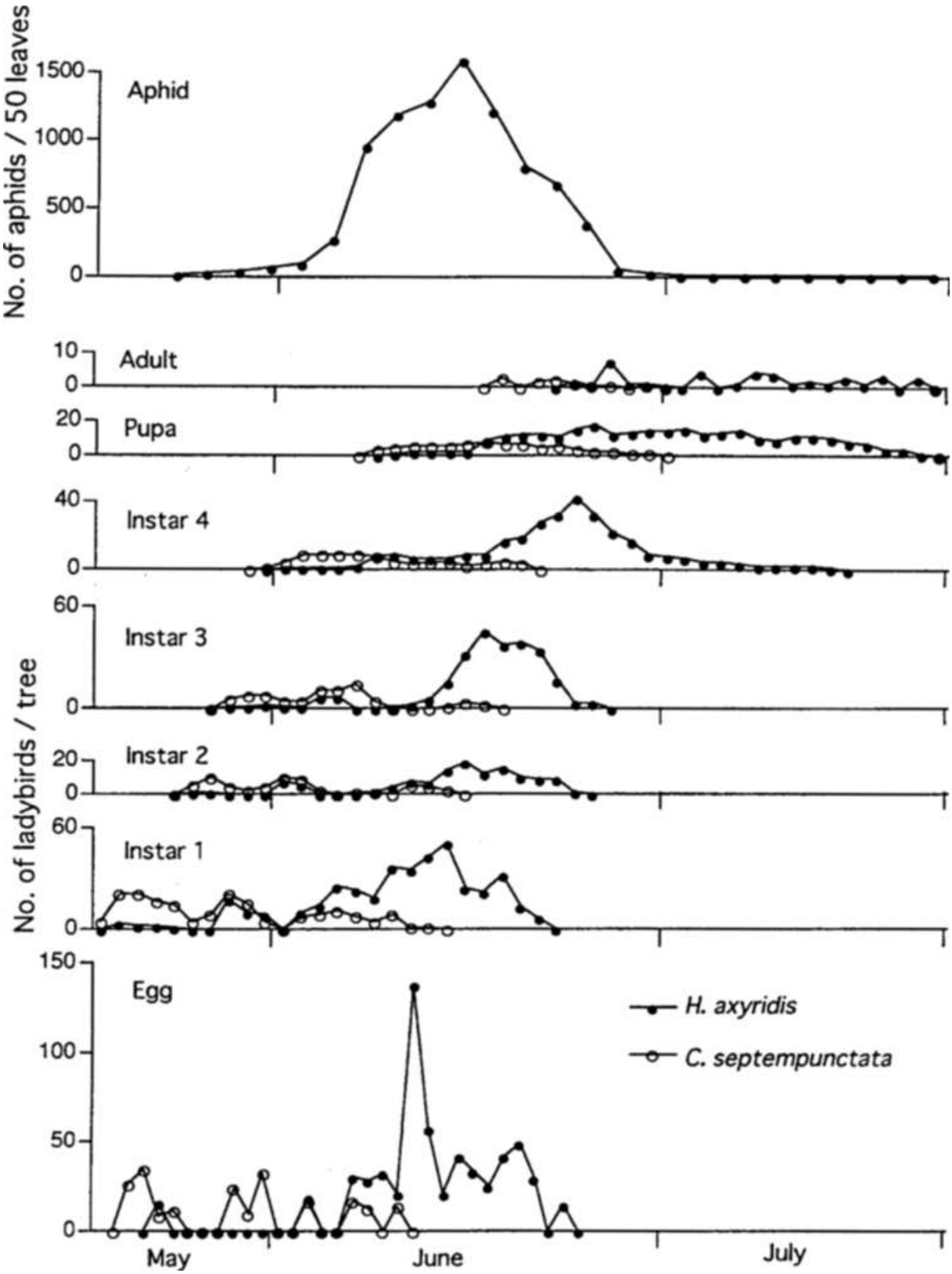


Fig. 2.7 Seasonal changes in the number of aphids and two species of ladybirds in 1994 from Yasuda & Shinya (1997).

2.5 Concluding remarks

When such life history measurements are done in a later period, care should be taken that the first oviposition period is included. This will allow for better comparison with literature and give a better indication of the actual period over which larval populations truly exist.

The measurement technique itself is quite good for purpose of practical usage. Though purely scientific arguments would point towards a more detailed and extensive daily observation schedule, the practical argument of time and energy weigh heavily. The experimental set-up here allowed a reasonably quick daily survey to be made with minimal materials required. A more detailed survey would likely have to have either 2 researchers doing the measurements or the surveys would have to be done every other day, rather than daily.

3 H. axyridis – E. balteatus intraguild predation experiment

3.1 Introduction

When studying nature in the Netherlands, it is more than likely that at a time, one will encounter the *Syphid* (Diptera) *Episyrphus balteatus*. This is a very common species that is easily recognisable by it's typical black banded pattern on it's abdomen on a gold-yellow background. Less known is that *E. balteatus* ' larvae feed on aphids as their primary food source. This makes *E. balteatus* a common choice in biological pest control.

Aphidophagous behaviour is well known in different groups. Various coccinellids and syrphid larvae are aphidophagous. These larvae are therefore also frequently food competitors. Such conflict will impact the community structure of both species (Hindayana et al., 2001). At the same time, many aphidophagous predators are not simply competitors. When competitive pressure becomes high, or if aphid populations collapse, aphidophagous insects are known to become predators of its competitors. This phenomenon is called Intraguild Predation (Pell et al., 2007).

When it comes to predation of other insect predators, size matters. Aphidophagous predator larvae increase considerably in size during their larval stages. The size of the larvae is directly important as a defence against predation as larger larvae are more able to physically overpower smaller larvae. In addition, they are more capable of pursuing and consuming larger prey. There are numerous other factors, such as the voracity and speed, that determine the success of the larvae in prey-predator interactions. This therefore determines the effectiveness of the predation by the predator in both intra- and interguild interaction. Younger larvae also have less developed and weaker defences against predation than older larvae. These defences can include direct defences, such as a hard shell, spikes or a coat of waxy material secreted by the insect's skin (Pell et al. 2008). There is also evidence of indirect defences inside the insects protecting it by the production of toxins (Ware et al., 2008). The reduced effectiveness of these defences in younger larvae is important when considering predation of these larvae. In general they will be more susceptible to predation and will be less successful in acting as a predator. As a larva matures, these defences become more important and allow it not only to survive, but eventually become a predator itself (Yasuda & Shinya, 1997; Yasuda & Onhuma, 1999).

Native species have evolved together in ecosystems for a longer time. In time, they have adapted to one-another. This has created an ecological balance between the species in these systems. It is well known that exotic species can severely upset such systems (Townsend et al., 2003). The Asian Multicoloured Ladybeetle (*Hamonia axyridis*) is an exotic species that threatens to disrupt a number of systems. It arrived

in the Netherlands in 2002 and was first found near the port of Rotterdam. From 2005 onwards, it has been increasing in registered numbers (Loomans, 2004). Due to its recent appearance, it is unlikely that the species has already adjusted to its new habitat.

Harmonia axyridis is an generalist aphidophagous predator. Though it feeds primarily on aphids, a lot is known about the interactions of *H. axyridis* with other Coccinellids, such a *Coccinella septempunctata, Adalia bipunctata*. In these interactions, *H. axyridis* is known to function as an intraguild predator (Yasuda & Shinya, 1997; Kalaskar & Evans, 2001; Ware et al, 2008; Burgio et al., 2002). This is important, because *H. axyridis* is known to have a major impact on other insect populations because of this behaviour as an intraguild predator on native populations in America (Evans et al., 2009; Mizell, 2007). In contrast, *H. axyridis* is not often the intraguild prey in intraguild interactions with other coccinellids. This can likely be contributed to the strength, size and voracity of *H. axyridis* larvae. In its native habitat in Asia, it was found to be the top insect predator within its guild (Pell et al., 2008).

In Europe, it is still uncertain what role the species will take in the food web. The voracity and size of its larvae suggest that interaction between *H. axyridis* and other species will most likely favour *H. axyridis* in terms of competition for resources and predation. As one of the intraguild species, *E. balteatus* would be a likely intraguild prey for *H. axyridis*. At the same time, *E. balteatus* is known to function as intraguild predator or prey dependent on size. *H. axyridis* first instar larvae are known to be susceptible to predation, making the possible prey (Yasuda & Shinya, 1997). Neither species are drawn from a population that has coexisted and coevolved, eliminating the possibility of direct functional adaptation to possible intraguild predation. *Harmonia axyridis* is known to predate on European aphidophagous coccinellids such as *Adalia bipunctata* and *Coccinella septempunctata*. Predation of *E. balteatus* would also therefore seem a logical consequence of interaction between these species.

This experiment aims to establish the exact result of interactions between *H. axyridis* and *E. balteatus*.

3.2 Hypothesis

H. axyridis is a notoriously strong intraguild predator and likely possesses additional defences against predation even during larval stages. Only the first instar (L1) of this species is known to be susceptible to intraguild predation. In addition, *H. axyridis* are known to be very voracious, both as a predator on aphids as intraguild predator, while *E. balteatus* is less reliant on this strategy. *H. axyridis* larvae also possess a high relative speed and is likely able to escape predation more easily than *E. balteatus*.

It is therefore suspected that only *H. axyridis* L1 larvae will be subject to predation by *E. balteatus*. At the same time *E. balteatus* larvae are likely only predated when the relative size of the larvae is closer together,

in either the L3 or L4 stage. The intermediate larval stage of *H. axyridis* is likely neither to be predator nor prey.

Due to the large requirement of food of later instar larvae, it would be likely to suspect that only a few days (less than 3) would be needed for complete predation of the intraguild population to occur.

3.3 Material and methods

3.3.1 Coccinellid cultures

The *H. axyridis* larvae used in this experiment were reared from eggs laid by wild-captured adults. These adults were captured near Wageningen and the Veluwe in the Netherlands and kept in outside cages through the winter. They were kept under constant conditions (25C±1, LD 16:8, RH 55 ± 5%) in the laboratory in 9cm vent less Petri dishes. The adults were fed an abundance of *Ephestia kuehniella* eggs, pollen and diluted honeywater (10%). Eggs laid by the adults were moved to a clean Petri dish where an abundance of *Ephestia* eggs was included to minimise cannibalism. When the larvae were in their 2nd instar, they were split into clean Petri dishes in pairs and again provided with an abundance of *Ephestia* eggs throughout their development.

E. balteatus larvae were reared on aphids feeding on *Brassica oleecerata*. These were aphids of the species *Brevicorne brassicae* (L.) and *Myzus persicae* (Sulzer) (Hemiptera: Aphidoidea). *E. balteatus balteatus* adults were obtained from the Koppert Company stock. The larvae were kept under greenhouse conditions (23C, natural daylight conditions) in plastic trays on aphid-infested leaves.

3.3.2 Experimental set-up

The cause of death for larvae during the experiment was recorded. It was assumed that the only cause of death for the larvae would be predation or cannibalism. The cause of death was therefore established by elimination. The pattern of feeding (bitemarks, sucked body fluids, etc) in the case of cannibalism was identified. Dead larvae that were not cannibalised, were assumed to have been predated by the other species, unless a clear specific cause of death (e.g. crushed by clumsy researcher). Experience with *H. axyridis* cannibalism was used as indication of the feeding behaviour by *H. axyridis*. The cannibalistic feeding pattern of *E. balteatus* was observed. Ten third instar (L3) *E. balteatus* larvae were released in a 9cm Petri dish. No food or moisture source was provided to the larvae. These larvae were then observed for a period of 48 hours and the pattern of feeding was recorded.

Four experimental treatments were set up for the actual Intraguild Predation experiment. In these treatments, all different larval instars of *H. axyridis* were combined with third instar larvae from *E.*

balteatus. The choice for only 4 treatments was based on the limited time and space available to conduct the experiment. In all treatments, five individuals of both species were used. They were placed in 9cm Petri dishes under constant laboratory conditions (25C±1, LD 16:8, RH 55 ± 5%). The *E. balteatus* larvae were reared under different conditions, but it was assumed that the larvae incurred no fitness effect from the laboratory conditions (personal communication with Martine Kos). In each treatment, the larvae were provided with ±30 aphids of the species *Acyrthosiphon pisum* (Harris) every day. It was assumed that this was sufficient to ensure larval survival, but would severely hinder larval development. The petridishes were then closed using Parafilm.

Every day, the number of their larvae and their developmental stages were recorded. Of all dead larvae, the cause of their death was recorded. The experiment was maintained and observed as long as there were living larvae present of both species. When either all larvae of a species were dead or had pupated, the experiment was stopped. It was assumed that neither *H. axyridis* nor *E. balteatus* had an interest for the pupae of the other species.

The number of dead larvae for each treatment was compared. A Kruskal-Wallis test one-way analysis of variance was used to establish if there was any statistical difference between treatments. This was done separately for both *E. balteatus* and *H. axyridis* mortality.

3.4 Results

Consumption of prey by *H. axyridis* was characterised by a lack of remains of the prey. This pattern was found for both *H. axyridis* prey as well as *E. balteatus* prey. A lack of remains, but a lesser number of larvae was therefore classified as cannibalism of predation by the *H.axyrids* larva. *E. balteatus* larvae always left remains of the victim. These were classified by the name 'corpse'.

In total, 20 arenas were made and observed. Not all treatments had the same number of repeats. An overview of the number of repeats and the conditions under which the treatment was ended has been included in table 3.1. The treatments were named after the larval instar of *H. axyridis* that was included in the experiment. In all variants a third instar (L3) larvae of *E. balteatus* was included.

table 3.1 – The conditions under which the observations were was stopped. The instar of the treatment refers to the larval instar of *H. axyridis* included in the experiment. In all treatments, third instar (L3) larvae of *E. balteatus* were included.

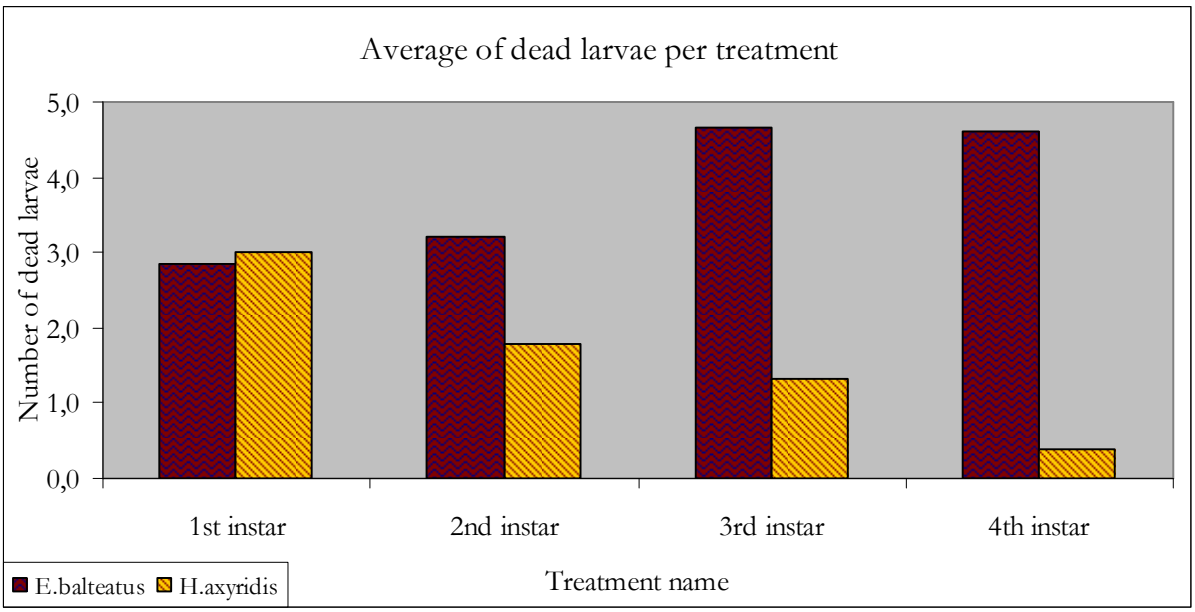
	Observation ending conditions							
	1st instar		**2nd instar**		**3rd instar**		**4th instar**	
	Number	**%**	**Number**	**%**	**Number**	**%**	**Number**	**%**
All *H. axyridis* pupa	1	14,3	1	20,0	0	0,0	1	20,0
All *E. balteatus* pupa	2	28,6	4	80,0	1	33,3	0	0,0
All *H. axyridis* dead	1	14,3	0	0,0	0	0,0	0	0,0
All *E. balteatus* dead	3	42,9	0	0,0	2	66,7	4	80,0

The number of dead larvae was observed and compared for the different treatments by species. An overview was given table 3.2 and fig 3.1. The statistical analysis showed that there was a significant ($p<0.05$) difference between the mortality figures of the treatments for *H. axyridis*.

table 3.2 – The average number of larvae that died per treatment and species. The instar of the treatment refers to the larval instar of *H. axyridis* included in the experiment. In all treatments, third instar (L3) larvae of *E. balteatus* were included. Species legenda: E = *Episyrphus balteatus*, H = *Harmonia axyridis*.

Average number of dead larvae		
Treatment	**Species**	**Number**
1st instar	E	2,9
	H	3,0
2nd instar	E	3,2
	H	1,8
3rd instar	E	4,7
	H	1,3
4th instar	E	4,6
	H	0,4

fig 3.1 – The number of dead larvae in the test at the end of the arena experiment. The instar of the treatment refers to the larval instar of *H. axyridis* included in the experiment. In all treatments, third instar (L3) larvae of *E. balteatus* were included. Species legenda: E = *Episyrphus balteatus*, H = *Harmonia axyridis*.



On average, in all combinations, over half the *E. balteatus* larvae did not survive to the end of the experiment. Of these death 5.4% was attributed to cannibalism. The *H. axyridis* larvae were the victim of IGP in 5.6% of the time. *H. axyridis* IGP and cannibalism can therefore be attributed to be the cause of 93.5% of all deaths.

3.5 Discussion

It becomes very clear from the experiment that *H. axyridis* is a strong competitor compared with *E. balteatus*. In nearly all experimental repeats, the *H. axyridis* larvae at least partially survived. The impact of *E. balteatus* larvae on the *H. axyridis* larvae was very limited, even in the earlier stages of development, while it would be expected that *H. axyridis* larvae would be more vulnerable in the earlier larval stages (especially L1 larvae). It is suspected that *H. axyridis* larvae have specific physiological defences against predation. In addition even *H. axyridis* L1-larvae move faster than *E. balteatus* L3-larvae and are therefore able to evade predation.

The smaller *H. axyridis* instars are not able to effectively predate the *E. balteatus* larvae due to their relative size. When the larvae grow bigger, they become more effective predators and therefore start to predate on the *E. balteatus* larvae. It is possible that the predation of *E. balteatus* larvae is regulated by the amount of food required by the *H. axyridis* larvae. Smaller larvae need less food. During the earlier instars, the diet of only a few aphids a day could be sufficient for its sustenance and development. Later instar larvae are more capable of intraguild predation due to a better relative size and a greater need for food.

The *H. axyridis* larvae showed no interest in the pupae of *E. balteatus*. This meant that pupae were left alone once fully formed, even by fourth instar larvae of *H. axyridis*. In situations were *E. balteatus* larvae were placed together with lower instar larvae, they could survive to the pupae stage. This is likely also related to the lower feeding rate of the lower instar *H. axyridis* larvae and therefore the great ability of *E. balteatus* to provide in its own food requirement. This meant that they could complete their complete development prior to the moulding of *H. axyridis* larvae into the third instar stage.

During the experiment, the length of the experiment was sometimes greatly extended because the filter paper on the bottom of the petri dish served as a barrier which the larger *H. axyridis* instars (third and fourth instars) could not cross. *E. balteatus* larvae are soft bodied and therefore more flexible than the hard bodied Coccinellids. This was not the case with earlier instar larvae of *H. axyridis* which would still fit through the small openings at the side of the filter paper. It is unlikely that this situation will occur in nature. *H. axyridis* larvae are equally able climbers on the bottom of leaves as they are on the top of these leaves. It is more likely that in nature the intraguild predation of these species will be lower, due to the

better spread of the larvae over the number of leaves. A brief survey of a *Tilia* branch showed a random, non-clustered distribution of both *H. axyridis* and *E. balteatus* larvae over the leaves.

Another important consideration is the development time and actual timing of the larval stages of both species. *H. axyridis* as an exotic species is not adapted (yet) to the phenological signals that indicate the start of aphid abundance, while *E. balteatus* and other species –such as *Adalia bipunctata* and *Coccinella septempunctata*- will have their life cycle sooner in the aphid season, benefitting of the highest population densities. This will also mean that intraguild predation predator-prey interactions between lower instar *H. axyridis* larvae with higher instar *E. balteatus* larvae are likely. As the relative stage of the larvae (and therefore their size) is important, this would suggest a better perspective for *E. balteatus* larvae surviving into their pupal stage, than when a parallel development is assumed. This is not currently surveyed, however, but could be included in a later study. As very little is known about *H. axyridis* in the Netherlands to begin with, such a study could incorporate a number of aphidophagous species.

Overall, the *H. axyridis-E. balteatus* interaction was typical for both species. *H. axyridis* was nearly completely immune to intraguild predation as prey, but acted strongly as intraguild predation predator when confronted with a food shortage. *E. balteatus* larvae were only predated by *H. axyridis* during the later larval instars of the latter, indicating that size is a determining factor for the intraguild predation relationship between the species. This 'size' matters hypothesis is also typical in intraguild predation relationships, but the strong position of smaller *H. axyridis* in this context is remarkable (Pell et al., 2007).

3.6 Concluding remarks

This experiment is too limited in scale to give a true evidence for the observed patterns, despite the clarity of these patterns. Repeating the experiment on a larger scale does not seem very useful, however. The pattern is following more or less what was hypothesised and it is very unlikely that the pattern will change if the number of repeats for this experiment is increased. It would be interesting, on a more general scale to observe this kind of behaviour in the field for multiple species of aphidophagous insects.

4 Larval tracks experiment

4.1 Introduction

The survival of juvenile insects to adults is usually very low, due to the size and quality of these insects in relation to the world around them. Maximising this survival -especially in early stages- is therefore very important for the overall success of an insect species. Adult insects can only have a limited effect on the fitness of its own larvae. The influence they do have is limited mainly to the ovipositions the eggs. A careful selection of oviposition site can be significant in the survival of the larvae by providing protection against environmental factors and biological dangers, such as predation. Another important factor is the clustering of eggs in patches, since patch-clustered eggs will receive mutual protection in microclimatic conditions (Yasuda & Ohnuma, 1999).

Aphidophagous ladybirds are no exception to this rule. Their larvae are usually mobile, but are land bound and need to be placed closed to a source of aphids. Female ladybirds must therefore select their oviposition site in close proximity to an abundance of aphids. These aphids are also important as a food source for the females themselves (Koch et al., 2003). When a ladybird larvae finds itself without the primary food source of aphids available to it, it will often resort to feeding on other species of insects. These species can include a range of other soft-bodied and scaled insects, and aphidophagous prey, including coccinellids. It is therefore important for the female ladybirds not only to find a patch where an abundance of aphids can provide sufficient food for its larvae, but also have as little competition as possible. Larvae are also known to be cannibalistic on eggs, especially during the first phases of their 1st instar (Yasuda & Shinya, 1997; Koch et al, 2003).

Harmonia axyridis is an Asian ladybird species that was introduced throughout the world as a biological control species. This was done in part because its larvae are easy to rear, very voracious and very resilient. In many places *H. axyridis* was released and adapted to the new habitats as an exotic species (Brown et al., 2008). As a wild species it has been found to have a negative impact on the abundance of local species (Koch et al., 2008; Mizell, 2007) . This is likely in part due to the larval competition of the *H. axyridis* larvae. *H. axyridis* larvae, however, are also known as ferocious intraguild predators, who will resort to such predation and cannibalism even under abundant food situations. *H. axyridis* is therefore likely to avoid a concentration of conspecific larvae will have a significant positive effect on larval fitness. In intraguild predation, older *H. axyridis* larvae are strongly favoured over the larvae of most European species (such as *Coccinella septempunctata* and *Adalia bipunctata*). The impact of *H. axyridis* on European species is therefore likely twofold. It is a strong competitor for food, but will also serve as an intraguild predator to further reduce larval survival in European species (Brown et al., 2008).

Ladybird larvae have a number of chemical defences. The chemical defences cause larvae leave odour tracks that can be detected by female ladybirds. These tracks are a combination of chemical compounds that is unique to each species. Larval tracks can therefore be used by these females as an indication of the presence of larvae on the patch (Magro et al., 2007). Young larvae are considerably smaller and weaker than large larvae. They therefore suffer the risk of predation and cannibalism by larger larvae. Eggs and young larvae will therefore suffer a negative effect to their fitness if ovipositioned near a concentration of other larvae. It would therefore be likely that females avoid such patches.

Due to the invasiveness of *H. axyridis* it is not entirely clear whether the larval tracks produced by European species are actually registered as ladybird tracks by the females of *H. axyridis* and vice versa. As these species have not co-evolved besides one-another, it is unlikely that they are specifically attuned to the other's larval track signature. This experiment will aim to establish the presence or absence of larval track attraction/repulsion on ovipositioning females of an exotic species (*H. axyridis*) and a native species (*A. bipunctata*).

4.2 Hypothesis

1. The presence of larval tracks of the conspecific larvae will reduce the number of females that lay eggs.
2. Both species will recognise conspecific larval tracks and will avoid ovipositioning on patches with these tracks
3. Due to a lack of co-evolution, neither species will recognise heterospecific larval tracks and will not actively avoid patches with these tracks.

4.3 Material and methods

4.3.1 Coccinellid cultures

The *H. axyridis* adults used in the experiment were all collected as adults from their overwintering aggregations at several location on the Veluwe (the Netherlands). At the end of November these adults were placed in outdoor cages during the winter and taken from these hibernation sites to be used in the experiment. Adults of *A. bipunctata* were taken from a stock culture at Leiden University. They were placed in 9cm diameter Petri dishes lined with a filter paper in male-female pairs. The beetles were fed ad libidum *Ephestia kuehniella* eggs, pollen and diluted honeywater (10%) in Eppendorf tubes stuffed with cotton wool to reduce the amount of evaporation. The dishes were cleaned twice a week, during which *Ephestia* egss, pollen and honeywater were renewed. Filter paper was renewed only when it was dirty or had the remains

of eggs on it. Eggs laid by the pairs were removed by crushing them and wiping them with a piece of paper. This was done on a daily basis to ensure the dishes were kept free of larvae. All beetles were kept at LD 16:8, 25C (±1C) with a relative humidity of 55% (±5%). The Petri dishes were spread out to provide maximum illumination.

Some of the eggs laid by the adults were split from the adults into separate dishes, rather than removed, to avoid cannibalism. These eggs were placed in a clean 9cm ventless Petri dish. Hatched larvae from these eggs were split out into individual Petri dishes. These dishes were labelled with the number of the larvae's parents. First instar (L1) larvae were kept in their hatch community and fed ad libidum *Ephestia* eggs. Older L1 and young second instar (L2) larvae were split into separate Petri dishes for further growth. This was done in both single larvae dishes as well as pairs, as no difference in growth and behavioural characteristics was observed. *H. axyridis* larvae were fed exclusively on *Ephestia* eggs. *A. bipunctata* larvae were provided with *Ephestia* eggs, pollen and honeywater, as they were found to have growth rate reduction when exclusively fed with *Ephestia*. The larvae were kept under the same conditions as the adults, but unlike the adults, they were stacked to preserve space. This was estimated to have no effect on larval development. Every day of research (except for weekends and Monday), the developmental stage was noted on a sticker on the Petri dish. In the experiment, only young larvae (1-48) hours were used to prevent pupation (which renders larvae immobile, reducing the amount of larval tracks). The labels of both larvae and adults were checked in the experiment for their number. This would prevent adults to be combined in the experiment with their own larvae.

4.3.2 Experimental set-up

The experiment was designed to show attraction or repulsion of larval tracks on ovipositioning females. This was done by creating arenas where larvae were released to contaminate one half with larval tracks, while being excluded from the other. After the larval contamination, female were released in the full arena and the oviposition locations were recorded.

The arenas were made in 9cm ventless Petri dishes. A filter paper was attached to the bottom with odourless double-sided sticky tape in the centre of the filter paper. This was done to prevent the larvae from crawling under the filter paper and to prevent the filter paper moving due to the movement of the larvae and adults during the experiment. A line was drawn with pencil in the middle of the filter paper and a cardboard separator was installed to prevent larval cross-over between the sides of the arena. See fig. 4.1 for a graphical overview of the set-up.

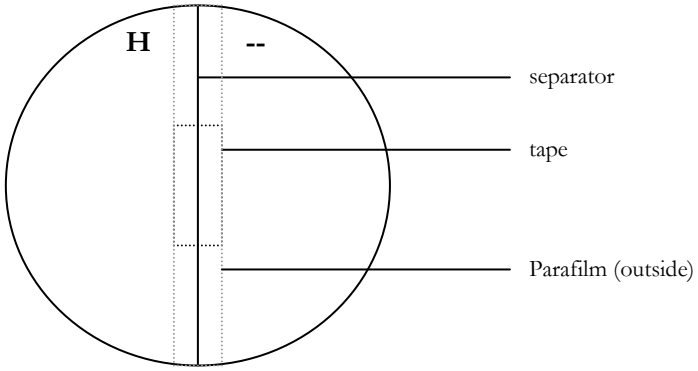


fig 4.1 The experimental set-up of the larval tracks experiment. The single-larval treatment for an *H. axyridis* is shown. The side marked with '—' has not been contaminated with larval tracks.

Six treatments and two controls were carried out in these arenas. These treatments can be split into three larval treatments and two different adult treatments, which were combined to give the total of 6 treatments. The two controls were adult treatments without larvae. The three larval treatments differed in the larvae released in the arena. There were two treatments in which one side of the arena was contaminated by larvae and one treatment in which both sides were contaminated with larval tracks from larvae (*H. axyridis* on one side, *A. bipunctata* on the other side). The difference between the two single larval treatments was the species of larva released in the arena.

To contaminate the arena sides, two fresh (1 – 48h since moulting) fourth instar larvae of a single species were taken from the culture and placed on one side the arena using a odour-coded brush. Care was taken to keep the other side of the arena clean of larvae. Fourth instar larvae were used to ensure maximum larval track strength, as they are large and mobile. Abundant *Ephestia* eggs were provided to prevent cannibalism between the larvae. The odour-coded brushes were used to prevent heterospecific chemical traces to be released in the larval side of the arenas. The arenas were then closed with Parafilm.

After 24 hours, the larvae, *Ephestia* eggs and faeces were removed from the arenas with the odour-coded brush. The cardboard separator was also removed. New *Ephestia* eggs and 25 aphids (*Aphis pisum*) were spread evenly in the arena to encourage oviposition. A female adult that had laid eggs in the 24 hours prior was then released in the arena. Care was taken to ensure no parents were placed in arenas where their own larvae had been released. After 1, 2, 3, 6, 8 and 24 hours, the arenas were checked. Any eggs laid since the last moment were marked on the arena using marker and their location was noted in relation to the larval-contaminated side of the arena. If the eggs were laid within 0.5cm of the separation line, this was also noted. This was done, because larvae of both species were likely to walk near edges and attempt to cross the boundary. A larger concentration of larval track chemicals could therefore influence the choice of the females for this location.

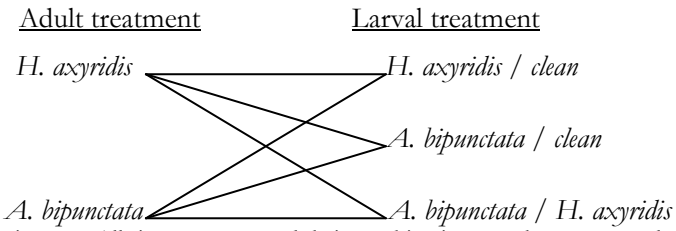


Fig. 4.2 All six treatments and their combinations. In the two control treatments, no larvae were released.

In the control treatment, adult females were released in neutral arenas. No larvae had been released in these arenas in the 24 hours prior. Unlike the other treatments, the location of eggs laid by these females was not recorded.

4.3.3 Statistics

The measurements were recorded to an Excell sheet and then introduced to the SPSS statistical package. Three classes of oviposition position were recorded: repulsion, attraction and neutral. The class was determined in relation to the conspecific larval tracks. A two-tailed Chi-square test for independence was ran on all three classes. An equal amount of cases was expected for this test. This was done for all six treatments. The inclusion of the neutral class was done to establish the importance of this neutral class in the general choice experiment. A second two-tailed Chi-square test for independence was ran for all treatments using only the 'repulsion' and 'attraction' classes to establish the choice between repulsion and attraction for ovipositioning females.

The percentage of females that laid eggs was calculated. A Kruskal-Wallis test between all groups was then ran to establish whether there was a significant difference between the larval treatments. If a signficiant difference was found, this was further refined by running Mann-Whitney tests between two specific groups. (Hemptime, 2002; Doumba, 1998).

4.4 Results

4.4.1 Three oviposition classes

All larval treatments had at least 16 non-neutral repeats (see table 4.1). A significant difference ($p<0.05$) was shown between all three classes for the single, conspecific larval and the dual-larval treatment with a *H. axyridis* female. The single, heterospecific larval treatment showed no significant difference between the three classes. When the treatments were considered for the *A. bipunctata* female, both heterospecific and conspecific single larval treatments were significantly different between classes. The dual larval treatment was not found to have significant difference between classes.

Table 4.1 The three different classes in relation to the side conatminated with larval track. In the two-sided larval treatment, the classes are related to the conspecific larval tracks. Repulsion = eggs laid on the side opposite from the conspecific larval tracks, attraction = eggs laid on the side of the conspecific larval tracks.

Adult species	**Larval treatment**	**Class**			**Chi-squared**	**Df**	**P**
		Repulsion	**Neutral**	**Attraction**			
H. axyridis	*H. axyridis / clear*	16	6	6	7.143	2	0.028*
	A. bipunctata / clear	9	4	9	2.273	2	0.321
	H. axyridis / A. bipunctata	18	1	5	19.750	2	0.000*
A. bipunctata	*H. axyridis / clear*	15	2	10	9.556	2	0.008*
	A. bipunctata / clear	12	1	4	11.412	2	0.003*
	H. axyridis / A. bipunctata	11	9	6	1.461	2	0.482

4.4.2 Two oviposition classes

The tests for the attraction or repulsion of *H. axyridis* were significant ($p<0.05$) for the treatments in which conspecific larvae were present (see table 4.2). In both these cases, *H. axyridis* was clearly repulsed by the conspecific larval track contaminated side of the arenas. No difference was found in repulsion or attraction in the arena without conspecific, but only heterospecific larvae ($p=1.000$). *A. bipunctata* was only clearly repulsed by the presence of only conspecific larvae. When heterospecific larvae were present, no preference was found.

Table 4.2 The two different classes in relation to the side contaminated with larval track. In the two-sided larval treatment, the classes are related to the conspecific larval tracks. Repulsion = eggs laid on the side opposite from the conspecific larval tracks, attraction = eggs laid on the side of the conspecific larval tracks.

Adult species	**Larval treatment**	**Class**		**Chi-squared**	**Df**	**P**
		Repulsion	**Attraction**			
H. axyridis	*H. axyridis / clear*	16	6	4.545	1	0.033*
	A. bipunctata / clear	9	9	0.000	1	1.000
	H. axyridis / A. bipunctata	18	5	7.348	1	0.007*
A. bipunctata	*H. axyridis / clear*	15	10	1.000	1	0.317
	A. bipunctata / clear	12	4	4.000	1	0.046*
	H. axyridis / A. bipunctata	11	6	0.474	1	0.491

4.4.3 Presence of female ovipositioning

Less females of *H. axyridis* laid eggs in the presence of conspecific larval tracks, when compared with the control (see table 4.3 and 4.4). No reduction was found in the presence of heterospecific larval tracks. The oviposition frequency of *A. bipunctata* was not found to be affected by the presence of larvae, either conspecific or heterospecific.

Table 4.3 The percentages of *H. axyridis* females that laid eggs during the larval track experiment for all larval treatments and the control.

Species female	Treatment	% eggs	N	Kruskal-Wallis Chi-square	Df	Sig
H. axyridis	*H. axyridis / clear*	52.8	53	9.050	3	0.029*
	A. bipunctata / clear	64.7	34			
	H. axyridis / A. bipunctata	60	40			
	Control	82.5	40			
A. bipunctata	*H. axyridis / clear*	77.1	35	1.267	3	0.737
	A. bipunctata / clear	73.9	23			
	H. axyridis / A. bipunctata	76.5	34			
	Control	84.6	39			

Table 4.4 The comparison between the different treatments. The treatment 'Larvae' is all larval treatments combined into one group, compared with the Control group.

Species female	Treatment comparison	Mann-Whitney value	Z-value	Sig
H. axyridis	*H/clear v.s. Control*	745.5	-2.966	0.003*
	A/clear v.s. Control	559.0	-1.734	0.83
	H/A v.s. Control	620.0	-2.209	0.027*
	Larvae v.s. Control	1924.5	-2.777	0.005*

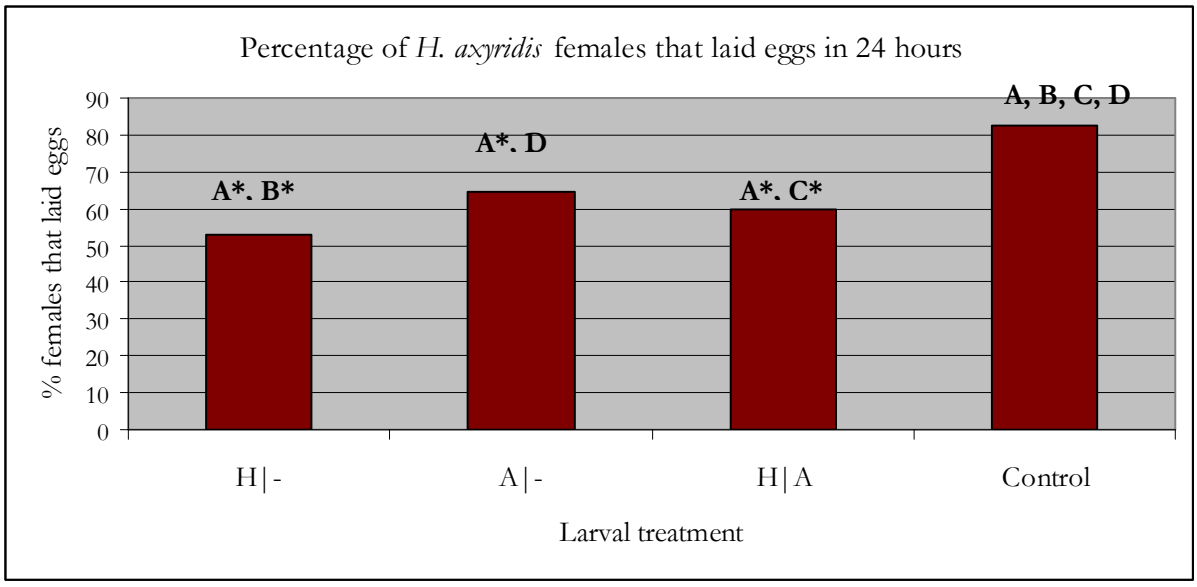


Fig 4.3 The percentages of ovipositioning *H. axyridis* females by treatment. H = *H. axyridis*, A = *A. bipunctata*.

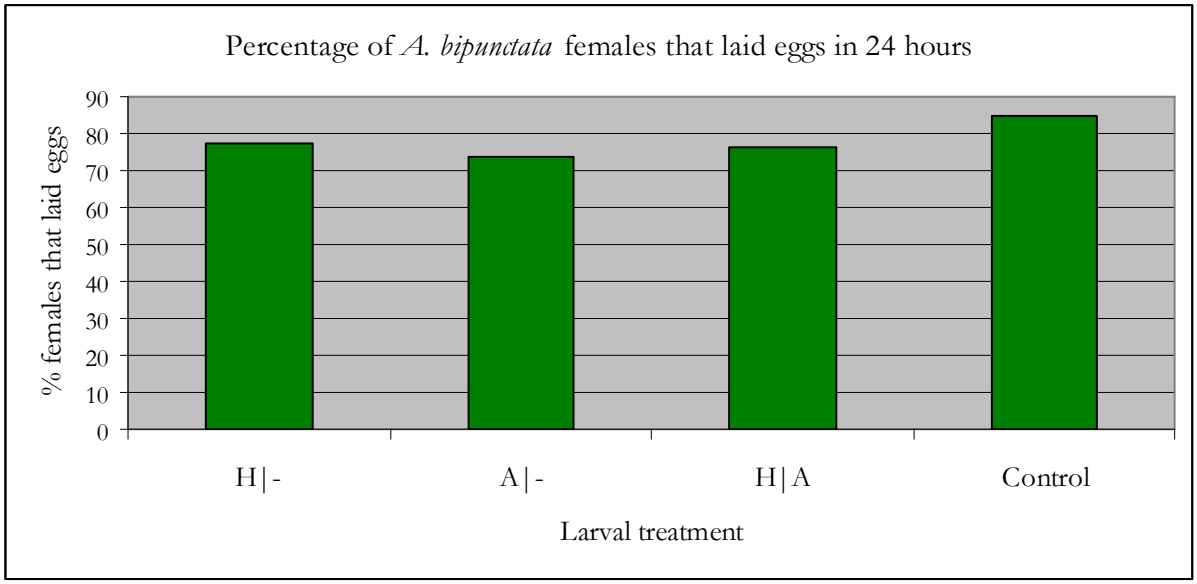


Fig 4.4 The percentages of ovipositioning *A. bipunctata* females by treatment. H = *H. axyridis*, A = *A. bipunctata.*

4.5 Discussion

The reaction of *H. axyridis* was typical of the repulsion and avoidance of larval track contaminated patches that would be expected. It was similarly clear that the larval tracks are species specific, as no response was found to the presence of heterospecific larvae. It could be possible that *H. axyridis* females do not recognise there tracks as belonging to a possible ladybird threat (though it is likely that they are recognised as coccinellid larval tracks (Margo et al., 2003)). The lack of a perceived threat cold be due to the superiority of *H. axyridis* as an intraguild predator and the fast development of its younger larval instars. It could also be argued that *H. axyridis*, because it lays its eggs relatively late (Yasuda, 1997), cannot effectively avoid ovipositioning on larval track contaminated patches, due to the abundance of larval tracks already present, similar to *Coccinella septempunctata* (Magro et al., 2003). This is further supported by the lack of oviposition reduction by *H. axyridis* females in the presence of *A. bipunctata* larval tracks, while this reduction was found in the presence of conspecific larval tracks.

Very much the same was found with *A. bipunctata*, but no specific repulsion was found in the case of dual larval track contaminated. This suggests that for some reason *A. bipunctata* does not respond to the conspecific larval tracks when also *H. axyridis* tracks were present. The females of *A. bipunctata* showed no response to the presence of just heterospecific larval tracks, while it has been shown to react to the presence of the heterospecific larval tracks of other native species (Magro et al, 2003). A response to conspecific larval tracks would therefore be expected, even in the presence of heterospecific larval tracks. The absence of this response gives the impression that the presence of the *H. axyridis* larval tracks diminishes the ability of *A.bipuncata* females to detect the conspecific larval tracks.

The lack of reaction to larval tracks is especially atypical for *A. binpunctata*. As mentioned before, a possible explanation could be the lack of recognision of the larval tracks of *H. axyridis* as a treat. This would a likely as neither population used here has shared territory. The larval tracks of the other species would therefore be considered alien. This would lead to the question whether the exposure to the other species would lead to a quick adaptation in larval track recognition and avoidance.

It would be possible to test this in a future study by including a combination of lab reared and wild cultures of *H. axyridis* and *A. bipunctata*. Europe is the only location where *H. axyridis* and *A. bipunctata* live together in the wild, but the *Adalia* used here were not caught directly from the wild. It would be expected that wild populations have experienced at least half a decade of contact with *H. axyridis* (Loomans, 2004). Running this experiment again with wild-caught individuals of both species could therefore provide evidence for adaption if an increased repulsion to *H. axyridis* tracks was detected. A similar effect could possibly detected in *H. axyridis* females.

In this study, two fifth instar larvae were found. Labrie et al. (2006) reported a 33% chance of an *H. axyridis* L4 larvae moulting to this fifth instar stage. These larvae were kept under the same environmental and feeding conditions as our larvae and were reared from the eggs of wild-captured individuals. It is not entirely clear how many larvae were present in the experiment, but the percentage in our culture was therefore clearly lower than found by Labrie et al.. The main difference between the larvae used by Labrie and our larvae, was the location from which they were gathered. Our larvae were taken from winter aggregations on the Veluwe, the Netherlands (Northern Europe), while Labrie collected his ladybirds in Quebec (Canada). The frequency of the appearance of a 5th instar larvae could be an interesting observation, for example for the non-genetic establishment of the origins of the *H. axyridis* populations in both the Netherlands and Quebec. Similarly, it could point to an early adaptation to the environmental and ecological conditions on both locations, if such ratio variations are not present within the native habitat of *H. axyridis*.

Despite the usage of young larvae, some larvae still pupated during their time in the arenas. The choice was made not to exclude these cases, though their pupation was noted. In most cases, this pupation happened towards the end of the 24 hour period (indicated by the activity of the larvae and the progress of the pupation). The 24 hour period was meant to ensure complete contaminated and it was assumed that the lost time during which the larvae was pupating did not affect the resulting larval patch contamination.

Concluding remarks

The choice of locations for the phenological experiment had a major flaw in the lack of *Tilia* trees in the city of Wageningen that were observed to function as an indicator of egg oviposition. The *Rosa nigosa* shrubs that were chosen to give this indication did not function well. Similarly, *Tilia* trees that were observed in the Arboretum did not show this larval development throughout the measurement period. When the transect is again set up in 2010, care should be taken to include at least 2 urban *Tilia* locations should such a location again be used for Life History observations (a recommended location would be the Marijkeweg trees). In addition, bi-weekly observations of these locations would be required to have an accurate starting point for these daily observations. When planning such an experiment, a period of 2 month observation should be expected at the very least. This will prevent observations to continue past the thesis time.

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Appendix I Phenological observations

Table I.1 The observations of coccinellids during the experiment organised by date.

Date	**Location**	**Observations**	**Notes**
6th of April	Blauwe Kamer	1x *Adalia bipunctata*	Observation by Renske, resting
	Schiphorst	1x *Coccinella septempunctata*	Resting
13th of April	Blauwe Kamer	4x *Harmonia axyridis*	Resting
20th of April	Blauwe Kamer	2x *H. axyridis*	Resting
	Schiphorst	2x *Coccinella septempunctata*	Resting, flying
	Rosa nigosa	22x *H. axyridis*	Resting, mating
		2x *A. bipunctata*	Mating
		1x *C. septempunctata*	Resting
27th of April	Blauwe kamer	1x *H. axyridis*	Resting
	Rosa nigosa	1x *H. axyridis*	Resting
4th of May	*Rosa nigosa*	1x *H. axyridis*	Resting
		1x *C. septempunctata*	Resting
		2x *H. axyridis* (L2)	Walking
	Grebbeberg	6x *H. axyridis*	Resting, mating
12th of May	*Rosa nigosa*	2x *C. septempunctata* (L3)	Walking
		1x *A. bipunctata* (L3)	Walking
		1x *H. axyridis* (L3)	Walking
19th of Mary	*Rosa nigoasa*	1x *Propylea quatuordecimpunctata*	Resting
		1x *H. axyridis* (PP)	
26th of May	Arboretum	17x *H. axyridis* (L1)	On *Tilia*
31st of May	Schiphorst	1x *C. septempunctata* (L4)	On *Poa*
	Grebbeberg	1x *H. axyridis*	On *Rubus*
15th of June	Grebbeberg	1x *H. axyridis*	On *Urtica*
		1x *H. axyridis* (L4)	“”
		2x *C. septempunctata* (L4)	“”
	Schiphorst	1x *H. axyridis* (P)	
		3x *H. axyridis*	Resting, mating