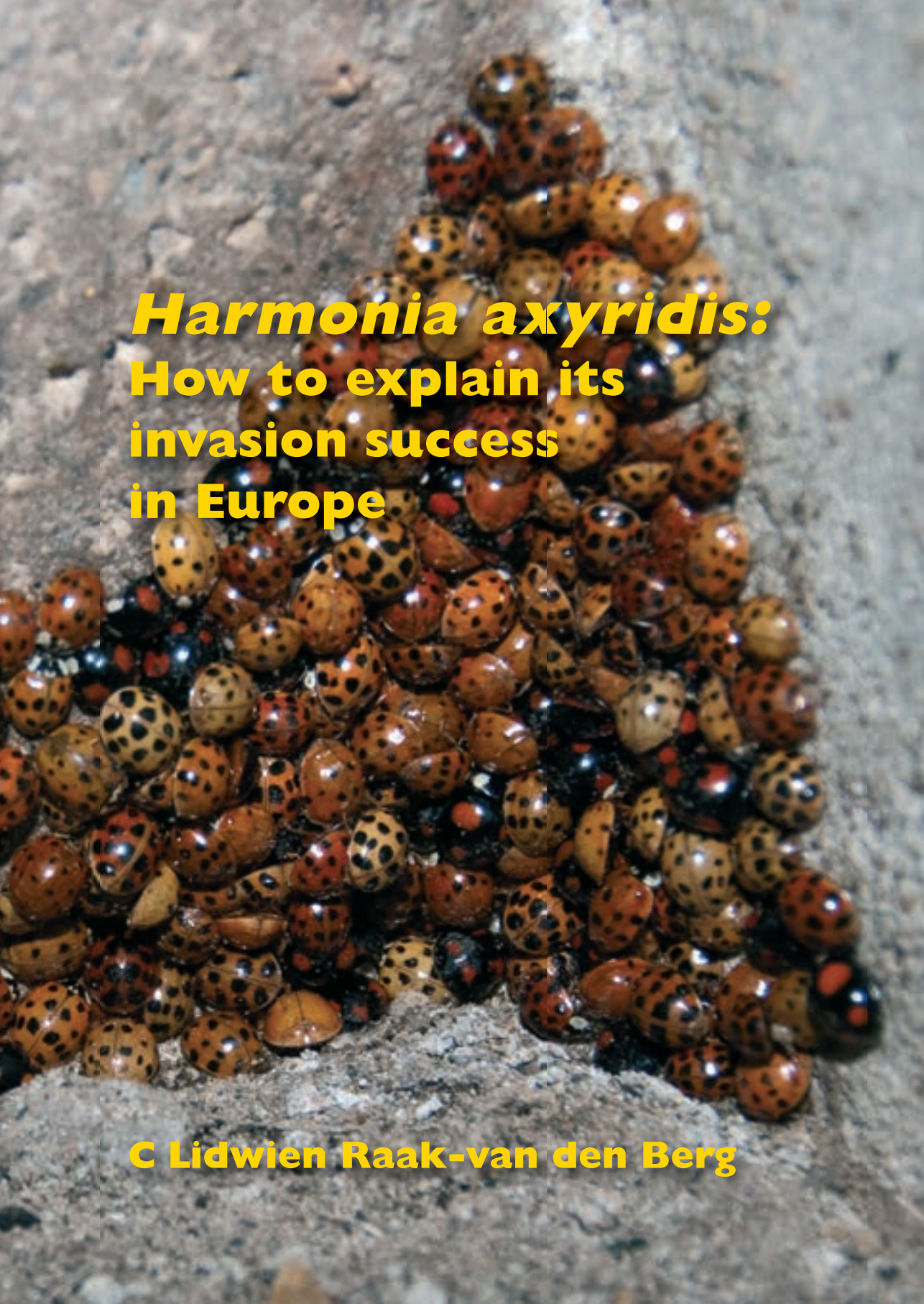

Harmonia axyridis: How to explain its invasion success in Europe

C Lidwien Raak-van den Berg

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This research was conducted under the auspices of the CT de Wit Graduate School for Production Ecology and Resource Conservation.

Harmonia axyridis: How to explain its invasion success in Europe

C Lidwien Raak-van den Berg

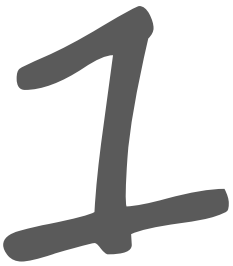
Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr M.J. Kropff
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 8 October 2014
at 1.30 p.m. in the Aula.

C Lidwien Raak-van den Berg
Harmonia axyridis: How to explain its invasion success in Europe,
222 pages.

PhD thesis, Wageningen University, Wageningen, NL (2014)
With references, with summaries in Dutch and English

ISBN 978-94-6257-103-7

Table of contents



General introduction

C Lidwien Raak-van den Berg

The multicoloured Asian ladybird, *Harmonia axyridis* (Pallas, 1773) (Coccinellidae, Coleoptera), is indigenous in Asia. Initially it was considered a promising exotic biological control agent against aphid pests (Anonymous 1996; Brouwer 1996; Horst and Tol 1996; Mertens 1996). After introduction, it established and spread rapidly through North America, Europe, and other parts of the world. It appeared to control aphids well, but also created problems. Because of its negative side effects it is no longer commercially available in most of Europe (Van Lenteren 2012) and is considered an Invasive Alien Species, i.e. "plants, animals, pathogens and other organisms that are non-native to an ecosystem, and which may cause economic or environmental harm or adversely affect human health. In particular, they impact adversely upon biodiversity, including decline or elimination of native species – through competition, predation, or transmission of pathogens – and the disruption of local ecosystems and ecosystem functions" (CBD 2014).

During my thesis research, I tried to determine the causes and consequences of the establishment and rapid spread of *H. axyridis* in the Netherlands. In this general introduction, I will first describe the ladybird *H. axyridis* as biological control agent, its current worldwide distribution, its negative side effects, and its biology. Subsequently, I will present the outline of this thesis.

Harmonia axyridis as biological control agent

Harmonia axyridis has been studied extensively during the last decades. In the period 1970—1990 experiments were conducted to study the ability of the beetles to control several aphid species and other soft-bodied insects in North America (e.g. Fye 1981; McClure 1987; Michaud 2000), Europe (e.g. Ferran et al. 1996; Katsoyannos et al. 1997a; Trouve et al. 1997), and Asia (Hukusima and Kondo 1962; Sun et al. 1996). In its native range, studies on its use as biological control agent and on methods of its mass production are still ongoing (Chen et al. 2012; Ruan et al. 2012; Nakayama et al. 2013).

Harmonia axyridis has a great voracity (Xue et al. 2009), which contributes to good aphid control. Because *H. axyridis* is also active at relatively low temperatures (15°C) (e.g. LaMana and Miller 1998; Soares et al. 2003), it can be used for aphid control in more temperate regions or when populations of native ladybirds decrease at the end of the summer and in autumn. *Harmonia axyridis* is able to survive on alternative food like pollen and sugar (Hukusima and Itoh 1976; Berkvens et al. 2008a), can develop on lepidopteran eggs (Schanderl et al. 1988; Chen et al. 2012) or an artificial diet (e.g. Okada and Matsuka 1973; Hukusima and Takeda 1975), has a high fecundity (e.g. Hukusima and Kamei 1970; Stathas et al. 2001), no obligate diapause (Hodek 2012b), and can be stored at low temperatures (Ruan et al. 2012). All these factors make mass rearing easy.

In the USA *H. axyridis* positively contributed to the control of the soy bean aphid (Fox et al. 2004; Mignault et al. 2006; Gardiner and Landis 2007) and of aphids in apple (Brown and Miller 1998), pecan (Mizell 2007), citrus (Michaud 2002a), roses (Snyder et al. 2004), corn, cotton, alfalfa, and hops (Michaud 2012). Augmentative releases in pine forests and bamboo have led to successful control of scale insects in Asia (Pervez and Omkar 2006). Information provided in many of the papers mentioned above leads

to the conclusion that *H. axyridis* is an efficient natural enemy for the control of aphids and other soft-bodied arthropods.

Distribution

Area of origin

Harmonia axyridis originates in Asia in a region bordered by Siberia in the North and southern China in the South and ranging from the Altai Mountains in the West to the Pacific Ocean in the East, including Korea and Japan. The ladybird occurs in a wide range of environments including continental, maritime, temperate, and subtropical climates (Poutsma et al. 2008; Brown et al. 2011b). Microsatellite, mtDNA, and CO1-gene data as well as differentiation in polymorphic morphological traits show that the native range of *H. axyridis* can be separated into a western and eastern zone: 1) Kazakhstan, Western Siberia, Altai, and Sayans and 2) the Amur region, the Far East, China, Korea, and Japan (Blekhman et al. 2010; Lombaert et al. 2011; Zakharov and Blekhman 2013).

Areas with intentional introductions

Harmonia axyridis has been introduced as biological control agent in many countries and in a wide range of crops and habitats (reviewed by Koch 2003; Pervez and Omkar 2006; Lucas et al. 2007). In North America, *H. axyridis* was first introduced in 1916 in the USA in California (Essig 1931) and Hawaii (Brown et al. 2011b). Subsequently, it was introduced in 14 different states in the 1970s and 1980s (Gordon 1985; Chapin and Brou 1991). *Harmonia axyridis* has also been introduced in South America in Argentina in the mid-1980s and late 1990s (Saini 2004; Poutsma et al. 2008) and in Chile in 1998 (Grez et al. 2010). The first introduction in eastern Europe was in Georgia (1927); followed by introductions in Uzbekistan, Ukraine, Kazakhstan, and Belarus (1950—1970) (Iablokoff-Khnzorian 1982; Poutsma et al. 2008). In western Europe it was introduced in France in 1982 to investigate biological control options (Schanderl et al. 1985; Coutanceau 2006) and kept in quarantine until 1990 when experimental field trials started. In 1995 the first commercial releases took place (Coutanceau 2006). In the Netherlands, the first trials with *H. axyridis* were performed in tree nurseries in 1995 (Brouwer 1996), and from 1996 onwards the ladybird was used in greenhouses (Mertens 1996) and in the field in tree nurseries and lettuce crops (Cuppen et al. 2004b).

Areas with invasions and establishment

As described above, *H. axyridis* has been released on several continents, and as a result it has established in many places. It took until 1988 before the ladybird established in the USA (Louisiana) (Krafsur et al. 1997), but by 2000 it had spread through a large part of northern America. In South America it was first reported in the wild from Argentina (2001) (Saini 2004) and Brazil (2002) (Almeida and Silva 2002), and by now it has established in several countries (Grez et al. 2010; Brown et al. 2011b). Although no information exists about intentional introductions, *H. axyridis* is also reported from South Africa (2001) (Stals and Prinsloo 2007), Lesotho (2008) (Stals 2010), and Kenya (2010) (Nedved et al. 2011).

The first European record of *H. axyridis* in the wild was in Germany in 1999 (Brown et al. 2008a). Since then, *H. axyridis* populations have rapidly increased in size and range and have now established in 27 European countries (Brown et al. 2008a; 2011b; Poutsma et al. 2008; Brown 2013).

In the Netherlands, the first record of this ladybird was a pupa found in Groesbeek in October 2002 (Cuppen et al. 2004b), in an area where it had not been released (AJM Loomans pers. comm.). Since then, its release for biological control came under pressure fuelled by new legislation and the emergence of negative environmental effects, and its use was forbidden in early 2005 (AJM Loomans pers. comm.). However, by 2006 *H. axyridis* had spread throughout the Netherlands (Brown et al. 2008a).

Several studies using climate and habitat matching predict a future expansion of *H. axyridis* covering a large part of the world (Koch et al. 2006a; Poutsma et al. 2008; Bidinger et al. 2012).

Negative side effects

In the invaded range *H. axyridis* is now regarded as an invasive alien species because its establishment caused many negative effects. The first negative consequences were reported in the 1990s (Kidd et al. 1995; Colunga-Garcia and Gage 1998; Cottrell and Yeargan 1998), and research has focussed on the negative impact of *H. axyridis* since. Many characteristics of *H. axyridis* are thought to contribute to its success in biological control, as well as to its invasion success.

In retrospect, we can conclude that the risks of introducing *H. axyridis* into new areas were not sufficiently evaluated before introduction. If a risk assessment evaluating the likelihood and magnitude of risks of non-target prey use, establishment, dispersal, and (in)direct effects on non-target organisms had been performed at the moment of introduction with the knowledge available at that time, this would have led to the conclusion that this species should not be introduced because of its polyphagy, high reproductive capacity, and large size in comparison with native ladybird beetles (Van Lenteren et al. 2008).

Impact on non-target arthropods

The establishment of *H. axyridis* has been associated with the decline of native coccinellid populations in urban, agricultural, and natural habitats in Europe (Brakefield and De Jong 2011; Roy et al. 2012) and in North America (Michaud 2002b; Alyokhin and Sewell 2004) and with a change in composition and dynamics of the native aphidophagous guild (Lucas et al. 2007).

In addition, *H. axyridis* may not only threaten members of the aphidophagous guild, but it may also have a negative impact on other native arthropods like the Monarch butterfly *Danaus plexippus* L. (Koch et al. 2003; 2006b), on non-pest aphid species (Koch and Galvan 2008), or on the weed biological control agent *Galerucella calmariensis* L. (Sebolt and Landis 2004).

Impact on humans

Not only insects are negatively affected by the invasion of *H. axyridis*. Humans also encounter this exotic ladybird, which can be a nuisance in their houses (Lucas et al. 2007, table 6.4) and can cause different kinds of inconvenience. In search of suitable hibernation sites the ladybirds fly in large swarms towards rocks, trees, or human-built structures such as houses (Obata 1986a). They may enter houses and form aggregations of up to several thousands of ladybirds (Nalepa et al. 2005; Wang et al. 2011). When temperatures rise due to central heating or the arrival of spring, the beetles become active, and large numbers start crawling and flying around inside buildings (Koch 2003; Majerus et al. 2006). Disturbed beetles release foul-smelling, yellow-orange reflex-blood, which causes stains on walls and soft furnishing (Huelsman and Kovach 2004) and may also cause allergic reactions (Goetz 2008). Ladybirds are even reported to bite humans (Kovach 2004). Sometimes the ladybirds overwinter in beehives, being a nuisance for the beekeepers but apparently not harming the bee colonies (Koch 2003).

Impact on crops

Harmonia axyridis may also interfere with food production. In autumn, *H. axyridis* adults move into orchards and vineyards and aggregate on and feed off ripening fruit (Lucas et al. 2007; Kögel 2012). This switch to frugivory is triggered by a decline of aphid populations after the harvest of crops (Kögel 2012), such as aphid-infested soy bean (Bahlai and Sears 2009). The beetles are difficult to remove from the grapes, and when they are disturbed or crushed during processing they release reflex-blood, which contaminates the wine. The alkaloids in the reflex-blood have an unpleasant odour and give the wine a bitter taste (Galvan et al. 2007; Kögel et al. 2012b). In the native range of *H. axyridis*, China, production losses are reported due to the ladybirds feeding on date flowers (Li et al. 1992).

Biology of *Harmonia axyridis*

Harmonia axyridis has a holometabolous life cycle, which is similar to that of other aphidophagous coccinellids, proceeding through the egg, four larval instars, pupal, and adult stages (e.g. Hodek 1973). All traits strongly depend on external conditions like diet and temperature. The data I present below relate to experiments done at temperatures of around 25°C; more details on life history characteristics and on a wider range of temperatures are presented in chapter 8.

During her lifetime a female can produce up to 3800 eggs (Hukusima and Kamei 1970) in batches of 20–50 (Berkvens et al. 2008b), which take three days to hatch (chapter 8). The larvae are elongated, black/dark grey, and have thick dorsal spines. The dorso-lateral sides of the second instar are orange. The third and fourth instar have a very clear orange colour on dorsal and dorso-lateral areas and, in case of the fourth instar, also on spines (Koch 2003). Development from larva to pupa takes about eleven days, and then adults take about five days to emerge (chapter 8).

Adults of *H. axyridis* have a convex, hairless body (Cuppen et al. 2004b) and are large (length: 5–8 mm) compared to other coccinellids (Kawauchi 1979; Iablokoff-Khnzorian 1982; Berkvens et al. 2008b). Pigmentation of labrum and prosternum of

males is light while that of females is dark and can be used for sex-determination in the field (McCornack et al. 2007).

The elytral colour pattern is highly polymorphic and heritable (Komai 1956) with relative frequencies varying with geographic location, time (Komai and Chino 1969), and season (Osawa and Nishida 1992). Four main colour forms are distinguished: red/orange (non-melanic) with 0 to 21 spots (f. *succinea*) and black (melanic) with two (f. *conspicua*) or four (f. *spectabilis*) red spots, or with a grid-like pattern (f. *axyridis*) (Majerus et al. 2006). Just after eclosion the elytra are pale orange, but the pattern appears within a few hours. The colour deepens over weeks or even months (Hodek 1973).

Non-overwintering adults typically live two to five months depending on temperature, diet, and other external conditions (chapter 8). In autumn adults migrate toward overwintering locations, where they aggregate in concealed locations (see also chapters 2, 3, and 4, this thesis). When temperatures rise in spring, the beetles mate and disperse to feeding areas (Koch 2003). Overwintering adults live on average another two months after emergence from hibernation, resulting in a total life span of 6 to 8 months (Jing et al. 2002; Bazzocchi et al. 2004).

Research objectives and thesis outline

The general aim of my thesis research was to understand the causes and consequences of the establishment and rapid spread of *H. axyridis* in the Netherlands. The specific research questions of this project were:

1. What is the life history of *H. axyridis* and does it help to understand the causes of its rapid establishment and spread in the Netherlands?
2. How does *H. axyridis* interact with native ladybird species and what are the consequences of its establishment?
3. What is the biology in the invasive range of *H. axyridis* compared with that in their area of origin? Are there differences that might explain their invasiveness in Europe?
4. Which natural enemies attack *H. axyridis* and might be used to control the ladybird in Europe?

Below I give an overview of the subjects covered in each chapter.

Chapters 2 and 3 focus on the mode of overwintering of *H. axyridis* and address the question whether *H. axyridis* overwinters in diapause or in a quiescent state. The intensity and length of the dormancy of *H. axyridis* is determined in **chapter 2**. To investigate this, hibernating individuals were collected and transferred to outdoor cages to continue overwintering. Intensity of dormancy was studied at two-weekly intervals by determining the pre-oviposition period and ovarian development of individuals that were transferred to long-day, warm conditions. If the intensity of dormancy turned out to be low, this could add to the explanation of the invasion success because low-intensity dormancy would lead to a rapid start of reproduction after hibernation, influencing population dynamics and competition with native species.

As continuation of the previous study, in **chapter 3** the hypothesis is tested that in northwestern Europe *H. axyridis* has a short and early period of real diapause starting at the end of October and shifts to a quiescent state in December. Ladybirds were sampled from their hibernation sites immediately after their migratory flights in October. Subsequently they were kept in outdoor cages, and then, after certain time-intervals, I measured the pre-oviposition time under optimal egg-laying laboratory conditions at 25°C at both short (D:L = 12:12) and long (D:L = 8:16) photoperiods.

Another aspect of overwintering is addressed in **chapter 4**. Survival during overwintering was assessed for wild *H. axyridis* populations in the Netherlands under natural and semi-natural conditions, with a focus on the potential influence on survival of location and orientation of ladybird aggregations at overwintering sites.

Many *H. axyridis* studies are performed under laboratory conditions, which impairs extrapolation to field situations. Therefore, life history characteristics during summer are studied under (semi-)field conditions (**chapter 5)**. Immature development and survival of *H. axyridis* and *Adalia bipunctata* L. (Coleoptera: Coccinellidae) on lime trees in cages were measured. In addition, the effect of co-occurrence of both species on survival and development was investigated.

Chapter 6 focusses on the intraguild predation behaviour of three ladybird species in the field: *Coccinella septempunctata* L. (Coleoptera: Coccinellidae), *A. bipunctata*, and *H. axyridis*. Predation behaviour of fourth instar larvae of these three species was investigated in semi-field experiments on small lime trees.

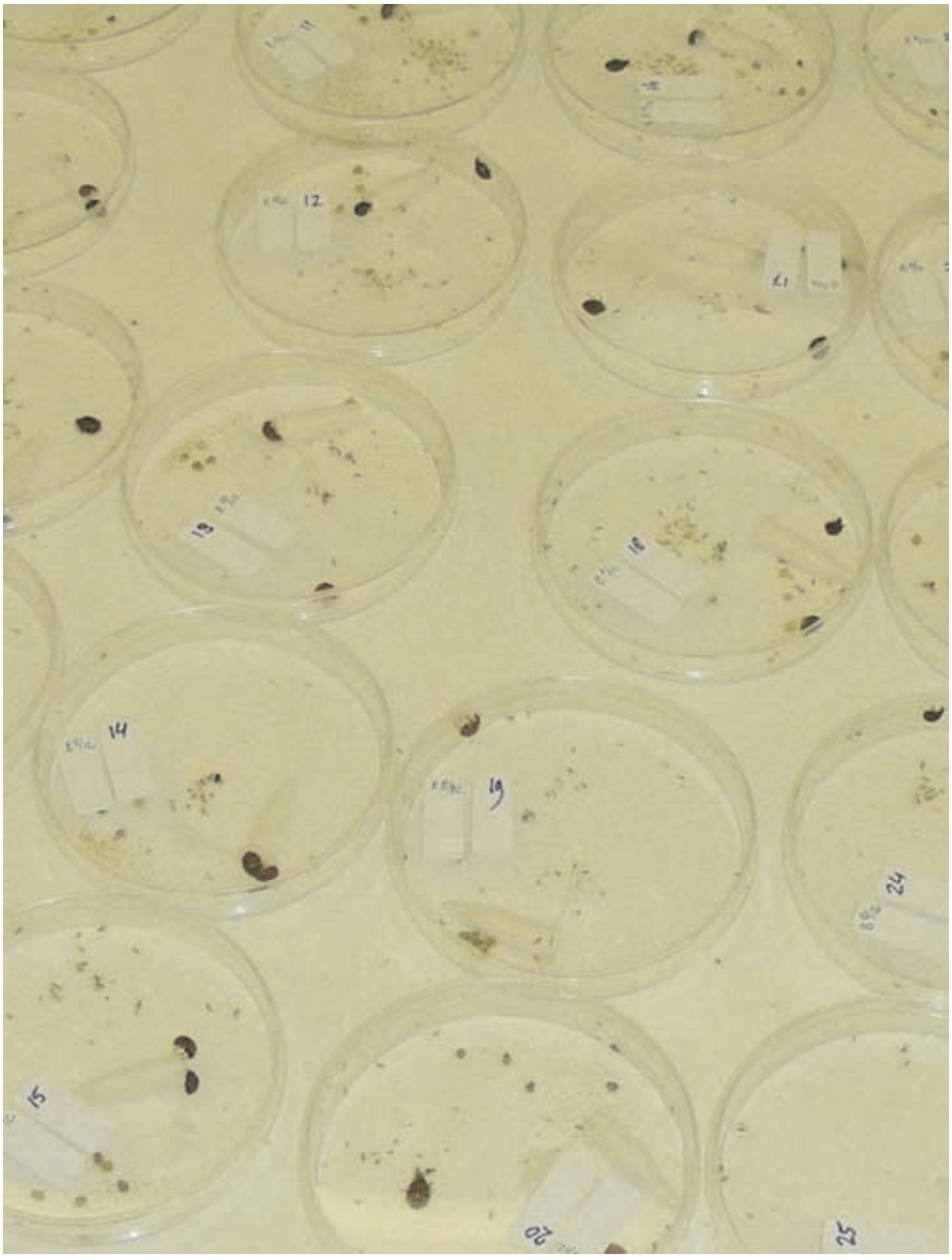
Chapter 7 summarises knowledge of natural enemies of *H. axyridis.* During the past ten years beetles were sampled in winter from hibernation aggregations and from spring through to autumn with illuminated screens at night. The beetles were checked for presence of natural enemies such as *Hesperomyces virescens* Thaxt. fungi, *Parasitylenchus bifurcatus* Poinar and Steenberg nematodes, *Coccipolipus hippodamiae* (McDaniel and Morrill) mites, and *Dinocampus coccinellae* (Schrank) parasitoids.

In **chapter 8** I first compare invasive and native Asian populations of *H. axyridis* to find differences in life history characteristics that may explain the invasion success of *H. axyridis*. Next, I compare life cycle parameters of native European ladybirds with those of *H. axyridis* to find potential explanations for the invasiveness of the latter.

Literature on life history characteristics of *H. axyridis* was collected and the main aspects that differed between studies (geographic origin, photoperiod, food, ladybird strain, and temperature) were identified. Subsequently, the effect of these variables on the life history characteristics of *H. axyridis* was determined with a meta-analysis. In order to address the second research question of this chapter I summarised information about life history characteristics of native European ladybird species and compared those with the characteristics of *H. axyridis*.

The results of the experimental chapters are summarised and discussed in **chapter 9**. An overview of life history traits that may explain the invasion success is given and suggestions for future research are made.

Acknowledgements
I would like to thank Joop C van Lenteren, Lia Hemerik, and Peter W de Jong for their comments on an earlier version of this chapter.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2

Mode of overwintering of invasive *Harmonia axyridis* in the Netherlands

BioControl (2011) 57: 71-84

C Lidwien Raak-van den Berg

Lia Hemerik

Peter W de Jong

Joop C van Lenteren

Abstract

After establishment of *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) in Europe, population densities of native ladybird species have decreased. The post-hibernation onset of female reproduction, a key characteristic influencing population dynamics and competition with related species, was studied. Hibernating individuals were collected and transferred to outdoor cages to continue overwintering. Every two weeks a sample of individuals was transferred to long-day, warm conditions. Intensity of dormancy was studied by determining the pre-oviposition period and ovarian development. Pre-oviposition periods were short throughout our observations, indicating that *H. axyridis* was not in diapause but in a quiescent state. *Harmonia axyridis* becomes active rapidly when temperature rises in spring but is not active earlier in the year than native species. Neither the mode of overwintering, nor the onset of spring activity can explain the invasion success of *H. axyridis*.

Introduction

The multicoloured Asian ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) was introduced as biological control agent in more than ten western European countries in the late 1990s (Brown et al. 2008a). The introduction of *H. axyridis* is now considered an unfortunate event because of its negative side effects on e.g. non-target species, fruit production, and human health (Koch 2003; Van Lenteren et al. 2008; De Clercq and Bale 2011). Since 2002 *H. axyridis* has spread rapidly through Europe (Brown et al. 2008a; Kenis et al. 2010), and it has currently established in twenty-six European countries and has been recorded in four other European countries (Brown et al. 2011b). The establishment of *H. axyridis* is associated with the decline of native coccinellid populations in urban, agricultural, and natural habitats in Europe (Adriaens et al. 2010; Brown et al. 2011a) and in North America (e.g. Michaud 2002b; Alyokhin and Sewell 2004). Several mechanisms contribute to the rapid spread of *H. axyridis* and its effects on native species. *Harmonia axyridis* is a polyphagous species that feeds on numerous aphid species (Koch 2003) and other soft-bodied insects (Tedders and Schaefer 1994). As food sources of endemic ladybird species largely overlap with those of *H. axyridis*, the latter competes for food with many native coccinellid species (Lucas et al. 2007). Its polyphagous behaviour and aggressive nature also make it a strong intraguild predator (Pell et al. 2008) while its high fecundity (e.g. Hukusima and Kamei 1970; Wang et al. 2009) and low larval mortality (Dmitriew et al. 2009) can contribute to rapid population increase. The details of some of these traits and their exact contribution to the invasion success of *H. axyridis* still have to be unravelled, like those of another key biological characteristic of *H. axyridis* with consequences for population dynamics and interspecific competition: the speed with which it can start reproducing after winter dormancy.

Harmonia axyridis overwinters as adult in a state of dormancy. In the dormant state metabolism is greatly slowed down and development and reproduction are suppressed (Tauber et al. 1986; Denlinger 2002). Dormancy can occur in any season and in this state an organism is able to survive a period of unfavourable conditions, such as a prolonged period of low temperatures. In insects dormancy in winter is also called hibernation (Tauber et al. 1986; Gullan and Cranston 2005). In the majority of cases, insect dormancy is diapause-mediated: long before the onset of adverse conditions, the insects react to environmental cues and start a period of dormancy (Tauber et al. 1986). During diapause, several processes are arrested (e.g. growth and ovariole development), additional energy is stored (increase of fat bodies), and protected sites are sought (Tauber et al. 1986; Denlinger 2002). These modifications in physiology, morphology, and behaviour are called the 'diapause syndrome' and are species-specific (Tauber et al. 1986). Reproductive diapause describes the cessation or suspension of reproduction in mature insects (Gullan and Cranston 2005). When conditions temporarily improve, diapause persists. Diapause requires specific stimuli for termination (Gullan and Cranston 2005). However, no specific diapause-terminating stimulus has been identified for most temperate-zone species that undergo an overwintering diapause (Tauber et al. 1986). In contrast, quiescence is a reversible state of very low activity and suppressed metabolism. Insects become quiescent when conditions become unfavourable. Quiescent insects remain highly

responsive to changing environmental conditions and can immediately resume development or reproduction when favourable conditions return (Tauber et al. 1986; Gullan and Cranston 2005).

After diapause has been induced by environmental cues, the diapause state is intensified until all or most species-specific symptoms of diapause are present. Diapause progression continues with a gradual decrease in sensitivity to the diapause-maintaining stimuli resulting in a gradual termination of diapause. Insects may sometimes enter a state of post-diapause quiescence if they encounter unfavourable conditions after diapause termination. In this case, the characteristic diapause symptoms remain, but the insects are in a quiescent state and can react immediately when favourable environmental conditions develop. The developmental stage in which insects enter diapause is species-dependent (Tauber et al. 1986); in *H. axyridis* it is the adult stage.

Depending on the climate, overwintering of *H. axyridis* in the Northern Hemisphere takes place between October and April and lasts three to six months (Tanigishi 1976). Shortening of day length and a general decrease in temperature induce hibernation in *H. axyridis* (Tanigishi 1976; Sakurai et al. 1993). On clear, still, and relatively warm days, beetles start flying towards overwintering sites (Tanigishi 1976; Obata et al. 1986; Nalepa et al. 2005). These migratory flights often cover several kilometres (N Osawa pers. comm. 2010) and are thought to be part of the 'diapause syndrome' (Tauber et al. 1986; Hodek et al. 1993; Hodek and Honek 1996).

The migratory flight of *H. axyridis* is directed by 'hypsotaxis': the beetles orient themselves towards the highest object on the horizon (Hagen 1962; Campan 1997). This can be a building, tower, pole, or anything else that forms a prominent, contrasting silhouette against the surrounding environment (Tanigishi 1976; Obata et al. 1986; Nalepa et al. 2005). At closer range alighting is stimulated by light colours (white and yellow) (Obata et al. 1986; Tedders and Schaefer 1994; Wang et al. 2011) and strongly contrasting lines (Nalepa et al. 2005). After landing, three types of behaviour can be distinguished (Tanigishi 1976; Nalepa et al. 2005): first, the beetles test whether the surface consists of an appropriate substrate; then they walk around in search of a local, dark site with an opening of at least 3 mm (Nalepa 2007); and finally they settle at those sites, where they form aggregations. In the final stage of the aggregation process the beetles appear to be influenced by volatiles emitted by conspecifics (Verheggen et al. 2007) and by faeces, residues, and dead beetles of the previous year (Tanigishi 1976; Nalepa et al. 2000). The ladybirds form clusters of up to several thousands of individuals in fairly exposed positions indoors, rendering overwintering a nuisance for house owners because of stains, smell, and allergic reactions (Koch 2003). In their native range the ladybirds leave the houses before the first frost and continue overwintering in mountain caves (Wang et al. 2011).

Despite the conspicuous migratory flights and the large clusters of beetles that are found in overwintering sites, few studies have focussed on the mode of overwintering of *H. axyridis* in the field, following cohorts throughout winter. Sakurai et al. (1992) indicated that overwintering of *H. axyridis* is diapause-regulated, since in their study ovaries remained undeveloped and corpora allata were small.

In Europe hibernation of *H. axyridis* was studied by Iperti and Bèrtand (2001). Like Sakurai et al. (1992) they found that hibernating *H. axyridis* had enlarged fat bodies and poorly developed ovaries. Moreover, they found that *H. axyridis* entered hibernation with an empty gut. Berkvens et al. (2008b) were able to induce a reproductive diapause in the fourth generation of a laboratory-reared population collected in the field in April 2005. However, they could not replicate this with individuals that were field-collected in June 2008 (Berkvens et al. 2010a).

The current study was performed to shed light on the overwintering modes of the exotic, but established, ladybird *H. axyridis* in the Netherlands. The intensity of its dormancy was studied by determining the pre-oviposition period of hibernating field-collected individuals that were transferred to long-day conditions.

Materials and Methods

Sampling of *Harmonia axyridis*

Hibernating adults of *H. axyridis* were collected from their hibernation sites at various locations in the Netherlands and at various dates during the winter of 2008–2009 (table 1). After sampling the beetles were transported to Wageningen in 1 litre jars with some wrinkled filter paper inside and covered with netting.

Effect of length of hibernation on pre-oviposition period

Overwintering conditions of sampled beetles

Overwintering beetles were kept at an experimental farm of Wageningen University. The jars with beetles were stored for about two weeks in an unheated shed under natural light conditions. During this storage period all beetles were counted in a climate room at 4°C.

On 21 November 2008 the beetles were placed in flat cages behind wooden shutters (120 x 75 x 3.5 cm) mounted on the south-facing wall of the barn (figure 1). These cages consisted of wooden frames (105 x 60 x 1.7 cm) covered with netting made of PVC-coated fibre glass. Behind each shutter a single cage was mounted. The netting was fastened with magnetic strips and Velcro to enable easy access to the beetles. A strip of aluminium above the cages kept out most rain: the shutters became moist with heavy rain, but no wet beetles were observed.

Three of the shutters with cages were placed left of the barn entrance and three to the right (figure 1). Inside the barn was a cooling chamber with thick isolating walls positioned against the wall with the “right-side shutters”, while a heater was located just at the inside of the wall that held the “left-side shutters”.

The samples from Steinhull, Posbank and Lunteren were each put in individual cages. The Kootwijk sample was large and was divided over two cages. The samples collected in Deelen, Kop van Deelen, Loenen, and Alphen aan den Rijn were small and were combined in one cage.

All beetles were put in the cages on the same day and within a time span of one hour. Each cage contained between 1981 and 2527 beetles. In two cages (one on the left side and the other on the right side) data loggers (MicrologPRO, Fourier Systems) recorded temperature and humidity every half hour.

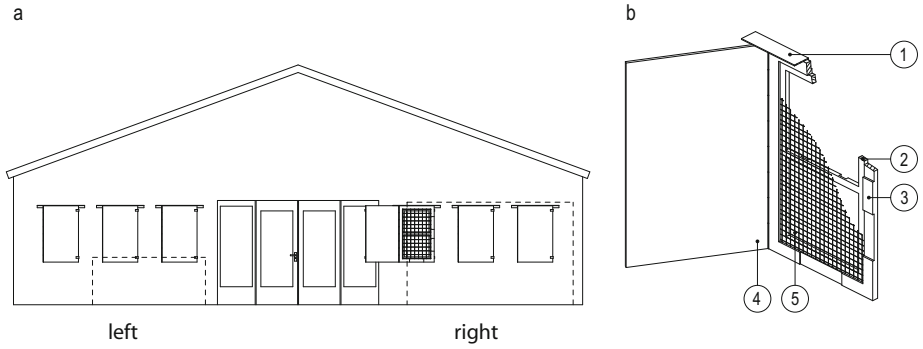

Figure 1 *a: Outdoor cages for hibernation of* Harmonia axyridis*. Cages are mounted behind shutters on a south-facing wall of the farm building. The heating (left) and cooling chamber (right) inside the farm building are marked with dotted lines. b: Detailed drawing of shutter with cage: 1) aluminium strip; 2) cage frame; 3) outer frame holding shutter; 4) shutter; 5) netting.*

Table 1 *Sampling locations, sampling dates an, sample sizes of aggregated hibernating* Harmonia axyridis *adults in winter 2008–2009, the Netherlands*

Location	Coordinates	Sampling date	Collection site	N
Deelen	N 52 3 53 E 5 53 23	7 Nov 2008	Outside: between wall and shutters	719
Kop van Deelen	N 52 3 37 E 5 53 54	7 Nov 2008	Outside: between wall and shutters	436
Posbank	N 52 1 45 E 6 1 18	7 Nov 2008	Outside: in folds of parasol	2061
Alphen a/d Rijn	N 52 7 27 E 4 40 2	11 Nov 2008	Inside: unheated room	547
Kootwijk	N 52 10 39 E 5 45 39	13 Nov 2008	Outside: between wall and shutters and between door and doorframe Inside: unheated and heated rooms	4604 242
Loenen, Ter Horst	N 52 7 29 E 6 1 45	13 Nov 2008	Inside: partly heated room	398
Loenen, Steinhull	N 52 6 2 E 6 0 23	13 Nov 2008	Outside: between wall and shutters and in corners of window frames	2530
Lunteren	N 52 5 39 E 5 37 3	13 Nov 2008	Inside: unheated attic	1984
Experimental farm	N 51 59 32 E 5 39 43	28 Nov 2008– 30 Mar 2009[a]	Overwintering in flat cages behind shutters during experiment	13521

[a] *After collection beetles continued overwintering at this location*

Determination of the length of the pre-oviposition period

Starting on 8 December 2008, samples of 40–60 beetles were removed from each cage before noon at biweekly intervals and transferred to a climate chamber (25°C, 16:8 L:D, 55±5 RH), where sex (McCornack et al. 2007) and colour were determined, and five pairs (male and female) were isolated from each of those six samples. If there were not enough males in a sample from a particular cage, a male from a sample from another cage was used. If a male died before the end of the experiment, it was replaced with a male from the same cohort, originating from the same cage, if possible. Each pair was put in a 9 cm diameter Petri dish lined with filter paper and provided with a mixed diet of frozen pollen, honey water (10%) in a small Eppendorf tube stuffed with cotton wool, eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), and live pea aphids (*Acyrthosiphon pisum* Harris (Hemiptera: Aphididae)). Every Monday and Wednesday new pollen, honey water, *Ephestia* eggs, and fresh aphids were added. Every Friday the beetles were transferred to clean Petri dishes and provided with fresh food. Pollen, honey water, and *Ephestia* eggs were given in excess.

The Petri dishes were checked daily for eggs. The pre-oviposition period was calculated as the number of days before oviposition of a cluster of fertile eggs. Eggs were separated from adults to avoid cannibalism and checked daily for hatching. A batch was considered hatched when neonate larvae had crawled out of their eggshells and were sitting on them or walking around. When the female did not start laying eggs within three weeks or when her eggs did not hatch, an extra male was added to check whether lack of oviposition was caused by female or by male sterility. The observation of a pair of beetles ended when the female died or when the first egg cluster hatched. In early spring beetles started to mate and to move around in the outdoor cages, which indicated that their overwintering period had ended. At that moment the experiment was terminated and all beetles were removed from the cages. The last sampling was done on 30 March 2009.

Determination of ovary development during hibernation

In 2008, at each sampling date part of the beetles collected at Kootwijk and Steinhull were frozen for subsequent dissection and observation of ovarian development. In 2009 beetles were sampled at five locations (Alkmaar, Houten, Kootwijk, Steinhull, and Zundert (chapter 4)) and continued overwintering at the experimental farm. Individuals were frozen for dissection at regular intervals. On the basis of earlier studies (El Hariri 1966; Sakurai et al. 1986; Okuda and Hodek 1989; Phoofolo and Obrycki 1995; Iperti and Bèrtand 2001) five stages of ovarian development were distinguished (table 2).

Table 2 *Developmental stages in ovarian development adapted after El-Hariri (1966), Sakurai et al. (1986), Okuda and Hodek (1989), Phoofolo and Obrycki (1995), and Iperti and Bèrtand (2001)*

Ovary stage	Description	Main characteristics
1	no eggs present	No eggs or follicles present in the ovarioles No or hardly any constrictions of the ovarioles No visible lighter coloured spots (indicating follicles)
2	1 egg per ovariole, transparent	First follicles are developing Follicles are transparent/whitish Ovarioles are constricted around the follicles
3	1–2 eggs per ovariole, yellow	Each ovariole contains 1 or 2 eggs Follicles become vitellinized/yellowish
4	2 eggs per ovariole, yellow	Each ovariole contains 2 eggs Follicles are vitellinized/yellow Size of follicles increases
5	3 mature eggs per ovariole, yellow	Each ovariole contains 3 mature eggs Eggs are large and yellow

Data analysis

The data on the length of the pre-oviposition period were analysed using Cox's proportional hazards model (Yano et al. 2005). The model permits estimation of the period until a particular event is likely to occur, and the effects of various factors on the length of the period can be analysed as well. A unique aspect of Cox's model is that it also takes into account censored situations, i.e. situations where the experiment is ended while the event has not yet occurred.

Cox's model can be written as follows:

$$h_0(t,Z) = h_0(t)\exp(\sum_{i=1}^{q} \beta_i z_i),$$

where $h_0(t, Z)$ is the hazard rate (here: tendency to oviposit) at time t with covariate values Z (z_i; i = 1,…, q) based on a baseline hazard rate $h_0(t)$ and the regression coefficients β_i (i = 1,…, q) that give the relative contribution of the covariates.

The rate at which the event occurs, given that it has not happened up to that moment (time t), is called the hazard rate. Cox's model assumes a certain baseline hazard rate, multiplied by a factor that accounts for the effect of the covariates. So the hazard at time t is expressed as a product of two quantities: the baseline hazard as a function of time and an exponential function involving fixed covariates. The main assumption of Cox's model is the proportional hazards assumption, meaning that the hazard rate of individuals with a certain covariate value is proportional to the hazard rate of individuals with another covariate value (Hemerik et al. 1993).

In this study, the event is the onset of oviposition after transfer of the beetles to warm, long-day conditions. If the female dies before oviposition, the observation is censored. The model assumes that the pre-oviposition period results from a basic oviposition tendency (i.e. the baseline hazard) and the effect of the covariate "time

since the experiment started" representing the length of the overwintering period. Data were analysed using SPSS (version 15.0.1.1, July 2007).

Results

Effect of length of hibernation on pre-oviposition period

The pre-oviposition period of 272 pairs of *H. axyridis* was determined. At the last sampling occasion on 30 March 2009, one cage was sampled five hours later than the other cages. Due to that delay, the beetles from that cage experienced more sunshine and a higher temperature than the other beetles. A preliminary analysis showed that this difference in treatment resulted in a significantly shorter pre-oviposition period and, for this reason, the data of these five pairs were excluded from further analysis. Three further observations were excluded from the analyses because the females died before any other female from the same sampling date had started oviposition. In total, 14 of the remaining 264 (5.3%) pairs did not oviposit at all and were censored.

We observed that the temperature in cages behind shutters on the left side was on average 3.3°C higher than on the right side throughout the experiment (figure 2). This consistent temperature difference significantly influenced the pre-oviposition period (Log-rank test, $\chi^2_1 = 4.373$, P = 0.037). Beetles hibernating in the warmer left-side cages tended to start oviposition earlier than those in the colder right-side cages, but the effect was not proportional within the model.

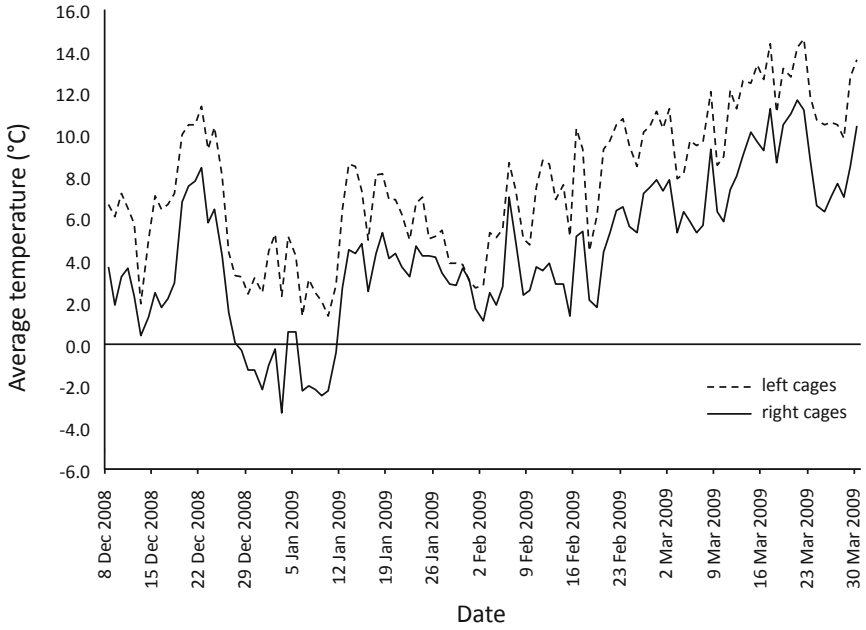

Figure 2 *Average daily temperature recorded in the cages*

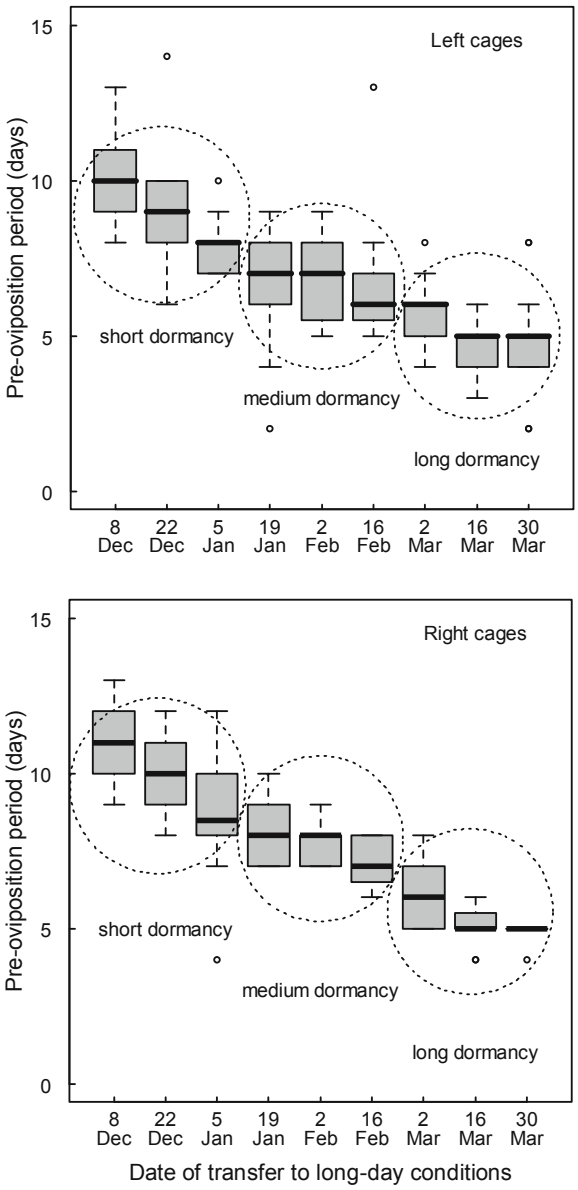

Figure 3 *Distribution of pre-oviposition periods of* Harmonia axyridis *females after transfer from outdoor hibernation cages to long-day conditions (25°C, 16:8 L:D). The pre-oviposition periods are grouped based on the length of the hibernation period of the beetles. The boxes represent the first to third quartile range with the median indicated by a line across the box. The whiskers represent roughly a 95% confidence interval for the difference between two medians. Outliers, which are all censored observations, are represented by open circles; some (6 for Left cages and 5 for Right cages) lie outside the plot range and are not shown.*

To analyse the effect of the timing of subsample transfer to long-day conditions on the pre-oviposition period, we used a stratified Cox's model to include this non-proportional temperature effect of left and right-side cages. The timing of subsample removal did not affect the length of the pre-oviposition period, and the effect was not proportional within the model. Figure 3 shows the median pre-oviposition period for each sampling moment, stratified for left and right. Based on this preliminary analysis the sampling moments were grouped into three periods with different responses: 1) short dormancy period: hibernation up to 5 January at the latest; 2) medium dormancy period: hibernation up to 19 January at the earliest and up to 16 February at the latest; and 3) long dormancy period: hibernation up to 2 March at the earliest and up to 30 March at the latest. The effect of the length of the dormancy period was tested with a Cox's regression model stratified for left and right cages.

The length of the dormancy period significantly influenced the pre-oviposition period (Cox's regression Wald test = 96.450, df = 2, $P < 0.001$). Beetles with a long dormancy period started oviposition after five days (median) regardless of whether they had hibernated on the left or right side. Beetles with a short dormancy period had a median pre-oviposition period of 9 (left) and 10 days (right). For beetles with a medium dormancy period the median pre-oviposition period was 7 (left) and 8 days (right) (figure 4). Colour morph did not significantly influence the pre-oviposition period.

Cox's model determines a hazard rate for all values of the categorical covariate (length of the dormancy period) for both strata (left and right). In this study the hazard rate was 'the oviposition tendency' i.e. the probability per time unit that a female started to lay eggs when oviposition had not yet started. The oviposition tendency increased when the dormancy period was longer.

The hazard ratio between beetles with long and short dormancy periods was 5.38, and that between beetles with medium versus short dormancy was 1.95. This means that the tendency to begin oviposition at any point in time once transferred to long-day conditions was almost twice as large for medium-dormancy individuals as for short-dormancy individuals and more than five times as large for long-dormancy individuals.

Determination of ovary development during hibernation

In total 214 of the frozen beetles of the 2008–2009 winter were dissected, 157 of which were female. Only four beetles, sampled on 30 March 2009, showed some restriction of the ovaries (table 2). These ovaries were still in stage 1 of ovarian development, as the restriction was not visible in all ovarioles and no follicle development could be seen. During the 2009–2010 winter, 436 beetles were dissected, of which 266 were female. Three beetles, sampled on 29 March 2010, showed restriction in some of the ovarioles and were in stage 1 of ovarian development. One beetle, sampled on 29 March 2010, showed ovarian developmental stage 2.

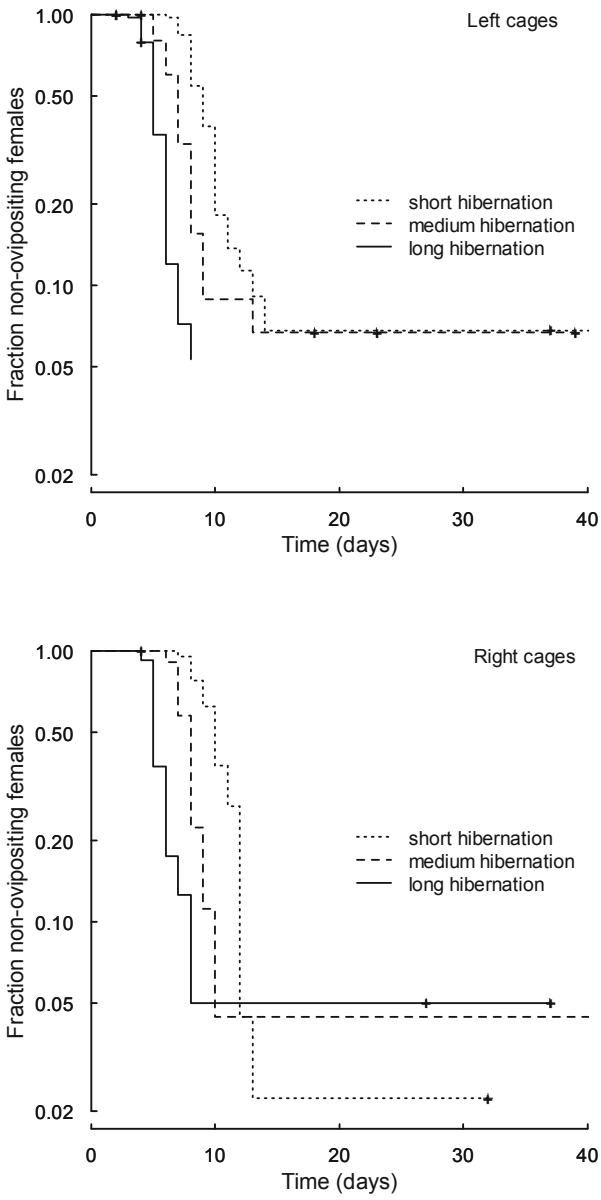

Figure 4 *Fraction of non-ovipositing females of* Harmonia axyridis *over time after transfer from outdoor hibernation cages to long-day conditions (25°C, 16:8 L:D). The data are grouped on the basis of the length of the hibernation period of the beetles (see text) and stratified for cages on the Left and Right side.*

Discussion

Diapause and/or quiescence?

Many studies have been conducted to unravel the mechanics of dormancy (see Tauber et al. 1986), and a number of studies has been done to determine whether *H. axyridis* shows diapause or quiescence (Sakurai et al. 1992; Ongagna and Iperti 1994; Iperti and Bèrtand 2001; Berkvens et al. 2008b). The results of these studies are not always consistent, and some are even contradictory, which we will illustrate below.

Insects in reproductive diapause will not react to an improvement of weather conditions by laying eggs, whereas insects that are in a quiescent state will start oviposition after transfer to favourable conditions. The intensity and development of the diapause can be estimated by the duration of the pre-oviposition period. Emerging adults of the *H. axyridis* summer generation show a pre-oviposition period of 5.25–11.5 days with a weighted average of 7.96 days when determined at 25°C, long-day conditions and with provision of prey (table 3). Compared to this, a pre-oviposition period that is at least twice as long can be considered as a sign of delayed oviposition due to diapause, according to Obrycki et al. (1983) and Hodek et al. (1989).

Table 3 *Pre-oviposition (pre-ovi) periods of emerging adult* Harmonia axyridis *determined at 25°C and under long-day conditions*

Pre-ovi period (days)	Population	Photoperiod (L:D)	N	Reference
10.1[a]	Field	natural day length	6	Hukusima and Kamei (1970)
11.5[a]	Field	natural day length	13	Hukusima and Kamei (1970)
7.4 [6–10][b]	Laboratory	16:8	15	Lanzoni et al. (2004)
9.8 (± 1.3)[c]	Laboratory	16:8	20	Schanderl et al. (1988)
10 [7–20][d]	Laboratory	16:8	16	Seko and Miura (2009)
7.5 [4–17][d]	Laboratory	16:8	24	Seko and Miura (2009)
7.2 (± 1.12)[c]	Laboratory	16:8	30	Stathas et al. (2001)
5.25 (± 0.87)[c]	Laboratory	16:8	24	Stathas et al. (2011)
7.92 (± 1.62)[c]	Laboratory	16:8	24	Stathas et al. (2011)
7.3 (± 0.7)[c]	Field	14:10	30	Wang et al. (2009)

Beetles were provided with a surplus of prey
[a]*mean* [b]*mean [range]* [c]*mean (± SD)* [d]*median [range]*

Two observations suggest that *H. axyridis* overwinters in reproductive diapause in the Netherlands. Firstly, in the current study the ovaries were regressed during the whole winter, like the ovaria of hibernating females in Japan (Sakurai et al. 1992). Sakurai et al. (1992) concluded that Japanese *H. axyridis* show true diapause because reproduction was completely suppressed, the corpus allatum was atrophied, and adults had a developed fat body.

Secondly, hibernation of *H. axyridis* is characterised by typical migration behaviour (Obata et al. 1986; Nalepa et al. 2005). In the Netherlands these migratory flights took place at the end of October in 2008. As *H. axyridis* arrives at its overwintering site long before true winter conditions set in, this behaviour could be seen as part of the diapause syndrome as defined by Tauber et al. (1986) and would indicate that *H. axyridis* enters diapause in the Netherlands at the end of October.

Moreover, two studies have been conducted where diapause could be induced in *H. axyridis*: in a population reared under outdoor conditions in southern France (Ongagna and Iperti 1994) and in a population reared in the laboratory for four generations in Belgium (Berkvens et al. 2008b).

In contrast to the above-mentioned information indicating existence of a reproductive diapause, the pre-oviposition periods of beetles transferred to long-day conditions in our study indicate lack of a reproductive diapause as they were evenly distributed around the mean and could not be separated into an early ovipositing group and a late ovipositing group as found in studies by Obrycki et al. (1983). The median pre-oviposition period of adults sampled on 8 December 2008 was 10 days. This falls within the range of the normal pre-oviposition period of the summer population (table 3), suggesting that *H. axyridis* was not in diapause in early December. Moreover, the adults also reacted to favourable conditions with a rapid resumption of oviposition. Thus, our finding of short pre-oviposition periods for beetles transferred from the field to warm laboratory conditions on 8 December seems to point towards quiescence rather than diapause. The explanation for these apparently contradictory observations might be that *H. axyridis* is only in diapause for a short period. If diapause of *H. axyridis* has a maximum duration of one month, beetles in the current study would have completed their diapause by the beginning of December and would already have shifted to a state of quiescence by the time the first sample to determine the pre-oviposition period was taken. This hypothesis is supported by Berkvens et al. (2008b), who found that it took aphid-fed adults from the fourth laboratory generation about one month to start oviposition under short-day (12:12 L:D) but warm (23°C) conditions.

Berkvens et al. (2008b) also showed that diet influences the pre-oviposition period. Females fed with *Ephestia* eggs had a pre-oviposition period twice as long as that of females fed with aphids (*A. pisum*). *Acyrthosiphon pisum* is a natural prey for *H. axyridis*, whereas *Ephestia* eggs are factitious prey (Hodek and Honek 1996). Factitious prey is reported to act as a diapause-inducing and diapause-maintaining stimulus (Hodek 1983). In the current study field-collected adults were used that were fed both aphids (*A. pisum*) and *Ephestia* eggs, so we did not expect a delay in egg laying due to a lack of optimal food.

Iperti en Bèrtand (2001) showed that hibernating adults in the mountains of south-eastern France, after transfer to long-day conditions (16:8 L:D, at 22°C), had a pre-oviposition period that shortened from January onwards. They concluded that *H. axyridis* was in a state of quiescence from that month onwards. The long pre-oviposition period of on average 16 days after transfer in December might indicate that these beetles were still in diapause. The pattern of one month of diapause and a switch to quiescence seems similar for the south-eastern French and the Dutch populations, which can be explained by similar temperatures in south-eastern France and the Netherlands during the study period. The long-term average temperature in south-eastern France (Antibes) in November is min 8.7°C and max 16.3°C (MétéoFrance 2010) while in the Netherlands in October 2008 the minimum temperature is 6.8°C and the maximum temperature 13.9°C (KNMI 2010). Although the average temperatures during winter are higher in south-eastern France than in the

Netherlands, these higher temperatures did not stimulate spring emergence to occur earlier than in the Netherlands.

At this state of our knowledge, we conclude that in the Netherlands, and perhaps more generally in western Europe, and maybe even in the area of origin of this species, *H. axyridis* enters a period of diapause of about one month. It shows characteristic migration and overwintering-site-selection behaviour before the start of winter and has undeveloped ovaries. In December diapause is complete, but oviposition does not occur because it is too cold for the beetles to feed and develop their ovaries. In the beach leaf mining weevil *Rhynchaenus fagi* L. (Curculionidae: Coleoptera) a similar mechanism has been observed (Bale 1979). In this study diapause ended in November/December, and post-diapause low temperatures actually produced the observed synchrony of emergence from hibernation and the budding of new leaves, rather than diapause being a synchronising mechanism between development and food availability. In the flesh fly *Sarcophaga bullata* Parker (Diptera: Sarcophagidae), too, pupal diapause is completed by the end of December while the low temperatures of the ensuing winter months suppress development and provide a synchronisation mechanism for uniform development when the developmental temperature threshold is reached in early April (Denlinger 1972).

As a result of a quite late start of the determination of reproduction, we measured the intensity of quiescence and the rate at which the beetles start reproduction after hibernation periods of different lengths rather than the depth of diapause. Determination of the pre-oviposition period during the month of supposed diapause is necessary to further support our hypothesis that *H. axyridis* shows a diapause of one month, followed by quiescence.

Diapause and quiescence in other ladybird beetles

Research with other ladybird species has been conducted in a way comparable to the present study. Obrycki et al. (1983) transferred *Adalia bipunctata* L. (Coleoptera: Coccinellidae) females from the field to several photoperiods in the laboratory. In October these females started laying eggs within 15 days under long-day conditions (16:8 and 14:10), whereas under short-day conditions it took more than 75 days to start oviposition. Transfer in December resulted in an average pre-oviposition period

Table 4 *Developmental thresholds (±SE when available in original paper) for* Adalia bipunctata, Coccinella septempunctata, Harmonia axyridis, *and* Hippodamia convergens*. Predation activity indicates the threshold for predatory activity. Thresholds have not been recalculated by the authors; data from original study or from paper referring to the original data are given.*

Lower developmental threshold (°C)					
Egg	**Pre-oviposition**	**Ovi-position**	**Post-diapause development**	**Predation activity**	**Reference**
Adalia bipunctata					
9.2 ± 1.2					Obrycki and Tauber (1981)
8.2					Sakuratani et al. (2000)
10.5					Honek and Kocourek (1988)
7.5					Hämäläinen and Markkula (1977) in Hodek and Honek (1996)
8.4 ± 0.3					Frazer and McGregor (1992)
			6.8		Obrycki et al. (1983)
Coccinella septempunctata					
10.2					Honek and Kocourek (1988)
7.3					Hämäläinen and Markkula (1977) in Hodek and Honek (1996)
11.5 ± 1.4	10.9 ± 0.6				Xia et al. (1999)
11.7					Hodek (1958) in Honek and Kocourek (1988)
6.8 ± 0.6					Obrycki and Tauber (1981)
9.2 ± 1.4					Frazer and McGregor (1992)
13.2					Butler (1982) in Hodek and Honek (1996)
13.6[a]					Butler (1982) in Hodek and Honek (1996)
11.1[a]	17.5[a]				Kawauchi (1983)
Harmonia axyridis					
9.76					Wang et al. (2009)
10.7					Schanderl et al. (1985)
11 ± 0.2					LaMana and Miller (1998)
10.2 ± 1.6	10.8 ± 1.7				Stathas et al. (2011)
11.4 ± 0.7	12.0 ± 2.6				Stathas et al. (2011)
	11.3				Stathas et al. (2001)
				9.3[b]	Soares et al. (2003)
				10.4[c]	Soares et al. (2003)
				10.4[d]	Soares et al. (2003)
				7.9[e]	Soares et al. (2003)
Hippodamia convergens					
9.7 ± 1.2					Obrycki and Tauber (1982)
10.1					Butler and Dickerson (1978) in Honek and Kocourek (1988)
9.5 ± 0.3					Frazer and McGregor (1992)
11.7 ± 0.9					Miller (1992)
11.3 ± 1.11					Miller (1992)
		12			Michaud and Qureshi (2006)

[a] *subspecies brucki* [b] *aulica adult* [c] *nigra adult* [d] *aulica larvae* [e] *nigra larvae*

of 10.8 days at the 14:10 photoperiod. At a short-day photoperiod some individuals started laying eggs within 30 days, while others showed a pre-oviposition period of more than 90 days. In March all females oviposited within 12 days regardless of the photoperiod. This study showed that in *A. bipunctata* long-day length can terminate diapause in the laboratory, while short-day length maintains diapause. The study also showed that in December the response to photoperiod had already diminished substantially, but that the beetles remained sensitive to a diapause-maintaining photoperiod (Obrycki et al. 1983).

Hodek and co-workers sampled *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) from hibernation quarters and transferred the beetles to long-day and short-day conditions. The difference in pre-oviposition period for the different photoperiods was large in October, while in December it could only be found in a part of the population, and in May it did no longer exist (Hodek et al. 1977; Hodek and Ruzicka 1979). In contrast, hibernating *C. septempunctata* populations from Greece and Spain did not show any response to photoperiod as the pre-oviposition period was similar under long-day and short-day conditions (Hodek et al. 1989; Hodek and Okuda 1993).

Our study shows that *H. axyridis* can rapidly become active when temperature rises. Thus, the start of reproduction and the rate of population growth of *H. axyridis* in spring will mainly be influenced by the availability and quality of prey and not by the mode of overwintering. Since *H. axyridis* is a polyphagous species, it is not dependent on the availability of a single prey species but can use many different kinds of prey that are available early. One could argue that this is an advantage over native species as it may contribute to rapid population growth. However, a comparison with the lower developmental thresholds of *A. bipunctata*, *C. septempunctata*, and *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) shows that the thresholds of *H. axyridis* are similar to those of *C. septempunctata* and *H. convergens* (table 4). The thresholds of *A. bipunctata* are even lower than those of *H. axyridis.* Moreover, phenological data from Belgium, the UK, and the Netherlands do not suggest that *H. axyridis* is active earlier than the other species (Adriaens et al. 2008; Waarneming.nl 2010; Brown et al. 2011a). Thus, neither the mode of overwintering nor the moment that adult beetles become active in spring can explain the invasion success of *H. axyridis*. We are presently studying overwintering mortality and interspecific competition behaviour of *H. axyridis* in order to find explanations for its quick establishment and large population growth in the Netherlands.

Acknowledgements

The following people have contributed to this study J Abma, Mr & Mrs Brandsma, AB Allema, SW Benedictus-van den Berg, CAJM van Boxel, T Bukhari, I Folkertsma, Mr & Mrs Huijsman, Y Jongema, Mr & Mrs Van der Kamp, Mr & Mrs Klok, AJM Loomans, K Oku, D Lucas-Barbosa, G Mekking, Paviljoen de Posbank, Profict, YT Qiu, MJ Raak, SE Raak, H van Roekel, A Siepel, and W van Tol.

3

Diapause and post-diapause quiescence demonstrated in overwintering *Harmonia axyridis* in northwestern Europe

European Journal of Entomology (2013) 110: 585-591

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Abstract

The Asian ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) is regarded as an invasive species in many parts of the world. In a previous study we hypothesised that *H. axyridis* enters diapause at the end of October and then shifts to a quiescent state in December in northwestern Europe. In the present study we test this idea of a short, early period of diapause by sampling beetles from their hibernation sites immediately after their migratory flights in October, subsequently keeping them in outdoor cages, and then, after certain time-intervals, measuring the pre-oviposition time under optimal egg-laying laboratory conditions at 25°C. We did this at both short (12L) and long (16L) photoperiods, since a photoperiodic response is an indicator of true diapause, rather than quiescence. A significant, albeit small, difference in pre-oviposition period between the two photoperiods, which disappears in December, corroborates our earlier hypothesis that the ladybirds are in a state of diapause until mid-December. Compared with that of native ladybirds the diapause of *H. axyridis* generally is relatively short and weak; moreover, it appears to have become shorter over the last decade. This flexibility in diapausing behaviour may be an important factor that contributes to the invasion success of *H. axyridis*.

Introduction

Dormancy is a state in which metabolism is greatly slowed down and development and reproduction are suppressed (Tauber et al. 1986; Denlinger 2002). In this state, an organism is able to survive a period of unfavourable conditions, such as a prolonged period of low temperatures, and the development of active life stages is synchronised with favourable conditions (Hodek 2012b). Dormancy can occur in any season; in winter it is also called hibernation (Tauber et al. 1986). Overwintering dormancy has been shown to be diapause initiated and diapause maintained in most insects (including all ladybirds studied (Hodek 2012a; 2012b)).

Diapause is induced by environmental cues, long before the onset of adverse conditions. During diapause, modifications in physiology (e.g. arrestment of growth and ovariole development), morphology (increased fat body), and behaviour (search for protected sites) occur. This so-called diapause syndrome is species-specific (Tauber et al. 1986; Denlinger 2002). Even when conditions temporarily improve, diapause will persist.

Over time, sensitivity to the diapause-maintaining stimuli usually decreases, which results in a gradual termination of diapause (Tauber et al. 1986; Denlinger 2002). In contrast, quiescent insects are in a reversible state of very low activity and suppressed metabolism: they remain highly responsive to changing environmental conditions, and when favourable conditions return they can immediately resume development or reproduction (Tauber et al. 1986).

When unfavourable conditions are encountered after diapause termination, most insects remain dormant and enter a state of post-diapause quiescence. In this case, the characteristic diapause symptoms remain, but the insects are in a quiescent state and can react immediately when favourable environmental conditions develop (Tauber et al. 1986; Hodek 2012b).

The developmental stage in which insects enter diapause is species dependent (Tauber et al. 1986); in *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) it is the adult stage. Diapause of the adult stages is also known as reproductive diapause as the most conspicuous aspect is the cessation or suspension of reproduction in mature insects (Hodek 2012a).

The multicoloured Asian ladybird *H. axyridis* is an efficient predator of aphids. It was first introduced as biological control agent in Europe in 1982, has established in the late 1990s and has rapidly spread since 2002 (Brown et al. 2011b). *Harmonia axyridis* is now regarded as an invasive species because it has a negative effect on non-target insect species, fruit production, and human health (Brown et al. 2008a; Koch and Galvan 2008; Van Lenteren et al. 2008; De Clercq and Bale 2011). In addition, the establishment of *H. axyridis* is associated with a decline in native ladybird populations (Michaud 2002b; Alyokhin and Sewell 2004; Brakefield and De Jong 2011; Roy et al. 2012). As a result of these harmful effects, the ladybird is no longer commercially available in most of Europe (Van Lenteren 2012).

Differences in life history characteristics between alien *H. axyridis* and native species of the same guild, determine, among other factors, the invasion success of the alien species. Winter survival of *H. axyridis* is high and therefore results in large spring populations, which contribute to a rapid population build-up (chapter 4). Depending

on the climate, overwintering of *H. axyridis* in the Northern Hemisphere takes place between October and April and lasts three to six months (Tanigishi 1976). The mode of overwintering of *H. axyridis* has been studied at several locations in Japan and Europe (Sakurai et al. 1992; Ongagna and Iperti 1994; Iperti and Bèrtand 2001; Berkvens et al. 2008b; Reznik and Vaghina 2011). These studies demonstrate that *H. axyridis* has the typical aspects of the diapause syndrome: ovaries of hibernating females are underdeveloped, the corpora allata are atrophied, and the fat body is developed (Sakurai et al. 1992; Iperti and Bèrtand 2001). Moreover, the beetles show characteristic migration and overwintering-site-selection behaviour long before true winter conditions set in (chapter 2, Obata 1986a; Nalepa et al. 2005). In laboratory studies Berkvens et al. (2008b) and Ongagna and Iperti (1994) were able to induce reproductive diapause in *H. axyridis* under short-day conditions; characterised by a prolonged pre-oviposition period, empty gut, regressed ovaries, developed fat body, minimal food uptake, low activity, and aggregation behaviour.

A further indication of diapause rather than quiescence is the presence of a photoperiodic response. In temperate climates, the diapause-inducing and maintaining stimulus for insects is often a short photoperiod (Hodek et al. 1977; Hodek and Ruzicka 1979; Obrycki et al. 1983; Tauber et al. 1986). The photoperiodic response can be quantified as the difference in duration of the pre-oviposition period under short- and long-day photoperiods. A loss of photoperiodic response indicates the end of diapause and, depending on the moment during overwintering that this occurs, the insects may enter an environmentally-maintained period of post-diapause quiescence (Hodek and Ruzicka 1979; Tauber et al. 1986; Hodek and Hodkova 1988; Denlinger 2002).

We hypothesized earlier that in northwestern Europe *H. axyridis* enters diapause at the end of October and then shifts to a post-diapause quiescent state in December (chapter 2). In this earlier study, we started our observations of the length of the pre-oviposition period of *H. axyridis* only from December onwards, and using only one light regime. Thus, we were not able to determine the pre-oviposition period, nor the ladybird's response to photoperiod, during the month of supposed diapause, which is assumed to start after the migratory flights in October. In this paper we test the hypothesis that *H. axyridis* enters a short period of diapause, followed by a period of quiescence, by monitoring pre-oviposition periods of beetles that were sampled immediately after the first migratory flights occurred. As an indicator of diapause, the presence of a photoperiodic response was determined by measuring the pre-oviposition period at both short and long photoperiods.

Materials & Methods

Sampling of *Harmonia axyridis*

Due to the very low numbers of *H. axyridis* found in comparison to previous years (chapter 2), it was not possible to restrict the sampling to one location and one date. Several sites used for hibernation by *H. axyridis* in previous years were monitored from September onwards, and the first aggregations of *H. axyridis* were sampled on 8 October 2010, one day after the first flight. After that, samples were taken on various dates and at various locations of which the precise date of migratory flights is not always known (table 1). From mid-October onwards, the maximum daily temperature

was around 12°C and from the end of October onwards the daily number of hours of sunshine was about one hour (figure 1) (KNMI 2010). This suggests that by the end of October no more migratory flights could have taken place, as other studies report that temperatures of about 18°C are needed to support migratory flights (Obata 1986a; Nalepa et al. 2005). The majority of the aggregations had been sampled by early November, shortly after the migratory flights.

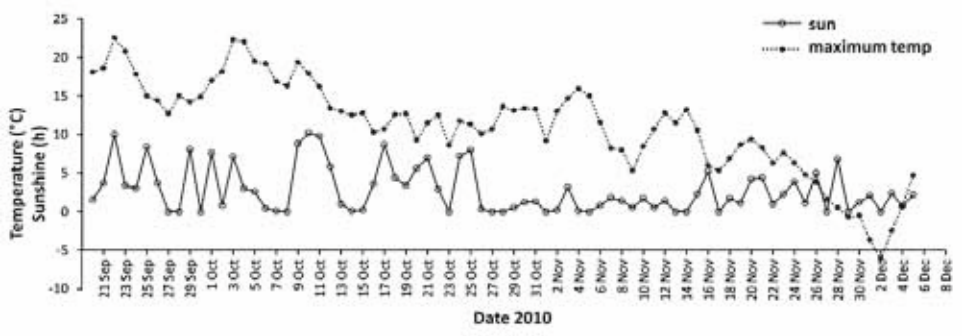

Figure 1 *Maximum daily temperature and total daily number of hours of sunshine in autumn 2010 in the Netherlands (KNMI 2010).*

Distances in the Netherlands are small and all locations are relatively close to one another: within a circle with a 75-km radius. Besides, the characteristics of the hibernation quarters (chapter 4) probably influence hibernation more than the location. To track any influence of locations, beetles from two locations were used for each time point (Tilburg and another location), from the moment onward that the large Tilburg population was collected.

After sampling, beetles were transported at ambient temperature (–2°C to 17°C) to Wageningen, the Netherlands. In Wageningen, beetles were allowed to continue overwintering in gauze bags in outdoor cages behind three wooden shutters on the right-hand side of the south-facing wall of the experimental farm building of Wageningen University (N 51 59 32, E 5 39 43) as described in chapter 4. The beetles at the locations not yet sampled by early November were still hibernating at the moment of sampling. This means that all beetles had been hibernating until they were used in the experiment. We therefore assume that the moment of sampling does not interfere with our results. Temperature was recorded every 30 minutes behind the middle shutter (MicrologPRO, Fourier Systems).

Table 1 *Sampling locations, sampling dates, and sample sizes of aggregated hibernating* Harmonia axyridis *adults in winter 2010–2011, the Netherlands. N = number of beetles used.*

Location	Coordinates	Aggregation reported	Sampling date	Sampling site	N	
					♀	♂
Alkmaar	N 52 37 57, E 4 44 35	End of October	20 Nov	Inside, on stone wall of unheated church tower	40	60
Alphen aan den Rijn	N 52 7 27, E 4 40 2	End of October	26 Oct	Inside, unheated room	76	56
Alphen aan den Rijn	N 52 8 33, E 4 39 22	End of October	29 Oct and 18 Nov	Outside, in steel window frames	44	44
Deelen	N 52 3 53, E 5 53 23		3 Nov	Outside, between stone wall and shutters	40	40
Deelen	N 52 3 37, E 5 53 54		3 Nov	Outside, between stone wall and shutters	40	20
Doesburg	N 52 0 57, E 6 8 9	End of October	3 Nov	Inside, unheated room	68	88
Kootwijk	N 52 10 39, E 5 45 39	7 October and 14 October	8 Oct and 15 Oct	Outside, between stone wall and shutters Inside, unheated and heated rooms	108	108
Tilburg	N 51 33 22, E 5 4 7		30 Nov	Inside, on stone wall of unheated water tower	227	227
Wageningen	N 51 58 53, E 5 40 20	9 October	9 Oct	Outside, on stone wall, while alighting	56	56
Winssen	N 51 53 11, E 5 41 22	End of October	3 Nov	Inside, unheated room and in window frames	12	12
Winterswijk	N 51 55 17, E 6 43 8	End of November	3 Dec	Inside, unheated room	11	11

Determination of the length of the pre-oviposition period

To determine the occurrence of diapause, the photoperiodic response − difference in duration of pre-oviposition period at two light-regimes − was measured. Groups of ladybirds were transferred to warm conditions (25 ± 1°C, 55 ± 5% RH) every week, and the time until oviposition of fertile eggs was recorded. Daily recordings under long-day conditions (16:8 L:D) started on 8 October 2010, one day after the first migratory flight, while those under short-day conditions (12:12 L:D) started on 2 November 2010, due to a technical problem with the climate room. From 21 January 2011 until 18 March 2011, transfers were made every fortnight.

After transfer, sex was determined (McCornack et al. 2007) and beetles were checked for visible infection with *Hesperomyces virescens* Thaxter (Laboulbeniales: Laboulbeniaceae) (Riddick and Schaefer 2005; De Kesel 2011; Haelewaters et al. 2012). Non-infected beetles were paired. Both under long-day and under short-day conditions generally twenty couples were observed per transfer date (with four exceptions). After 3 December half of the couples per transfer date originated from Tilburg and the other half from one of the other locations (table 1). Each couple was put into a 9 cm Petri dish and treated as described in chapter 2. Additionally, a folded strip of filter paper was added as substrate for oviposition, and aphids (*Acyrthosiphon*

pisum Harris (Hemiptera: Aphididae)) were given daily. Aphids were reared on *Vicia faba* L. (Leguminosae) and provided by Koppert Biological Systems, Berkel en Rodenrijs, the Netherlands.

Petri dishes were checked daily for oviposition. To avoid cannibalism, eggs were separated from adults and checked daily for hatching. As soon as neonate larvae had crawled out of their eggshells, a batch was considered hatched. If a female did not start laying eggs within three weeks or if her eggs did not hatch, an extra male, originating from a fertile pair, was added to exclude male sterility. Observation of a female ended when the female died or when the first egg cluster hatched. If a male died before the end of the experiment, it was replaced with another male.

Statistical analysis

The time until the start of oviposition after transfer to 25°C was analysed using survival analysis (chapter 2). Survival analysis is a method that allows for censored data – observations that are terminated before a certain critical event occurs – to be included and analysed, too. In this case, the critical event was oviposition. By using survival analysis in this study, the information that the female did not oviposit during her life is taken into account as the minimum time needed before the start of oviposition. If non-reproducing females were excluded from the analysis, this would result in an underestimation of the time until oviposition. The effect of photoperiod and dormancy length on the differences in the onset of oviposition were analysed with Kaplan Meier's Log rank test using PASW Statistics (18.0.3, 9 Sept 2010).

Results

The pre-oviposition period was determined for 722 pairs of *H. axyridis* (314 short-day and 408 long-day). In total 18 (short-day) and 31 (long-day) females did not oviposit at all, and those observations were censored.

As shown in figure 2 all locations (including Tilburg for which an extended record is available) show a similar trend: in the beginning, longer pre-oviposition periods are measured under short-day conditions than under long-day conditions; a difference which disappears in the course of the experiment. Therefore, results were pooled over locations.

The results are clustered in four groups with different dormancy lengths: 1) group I: transfer up to 12 November; 2) group II: transfer from 19 November up to 24 December; 3) group III: transfer from 31 December up to 4 February; and 4) group IV: transfer from 18 February up to 18 March.

The median pre-oviposition periods for the four groups under short-day conditions were 11, 10, 7, and 4 days respectively, and under long-day conditions 9, 8, 7, and 4 days respectively (figure 3).

The length of the pre-oviposition period under short-day conditions did not differ between groups I and II. The groups III and IV significantly differed from each other and differed from both group I and group II (for all significant pairwise comparisons: Log-rank tests with Bonferroni correction; confidence level 0.05/6, $\chi^2_1 > 24.4$, $P < 0.001$). The length of the pre-oviposition period under long-day conditions did not differ between groups I and II either. As under short-day conditions, the groups III and

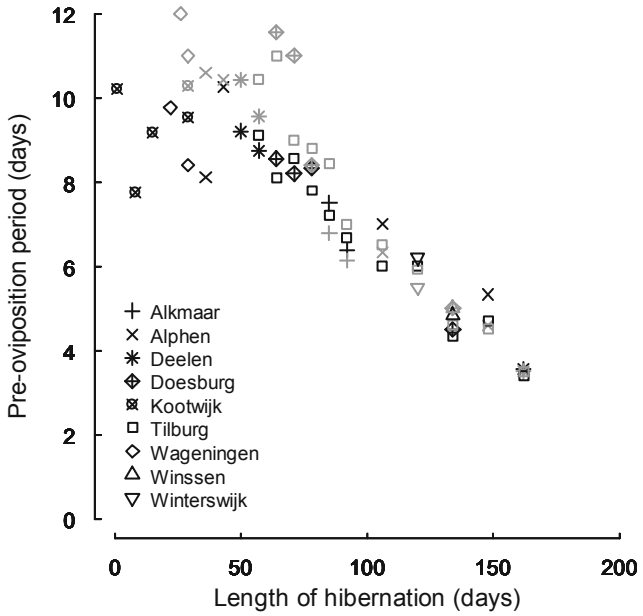

Figure 2 *Median pre-oviposition period of* Harmonia axyridis *females after transfer from outdoor hibernation cages to conditions representing long-day (25°C, 16:8 L:D) (black) and short-day (25°C, 12:12 L:D) (grey). The X-axis represents the length of the hibernation period (time between migratory flights and transfer). Median is given per population origin (indicated by different symbols).*

IV differed from each other and from both group I and group II (for all significant pairwise comparisons: Log-rank tests with Bonferroni correction; confidence level $0.05/6, \chi^2_1 > 34.0$, $P < 0.001$). Thus, from the end of December onwards (i.e. group III and IV), the length of the dormancy period significantly influenced the length of the pre-oviposition period: the longer the dormancy period, the shorter the pre-oviposition period (figure 3).

Analysis of the effect of photoperiod on the length of the pre-oviposition period stratified for dormancy group, showed that the pre-oviposition period under short-day conditions was significantly longer than under long-day conditions in dormancy groups I and II (pairwise log-rank tests per stratum: group I $\chi^2_1 = 10.1$; $P = 0.002$; group II $\chi^2_1 = 13.1$; $P < 0.001$). In groups III and IV short- and long-day conditions did no longer lead to significantly different pre-oviposition periods (figure 4).

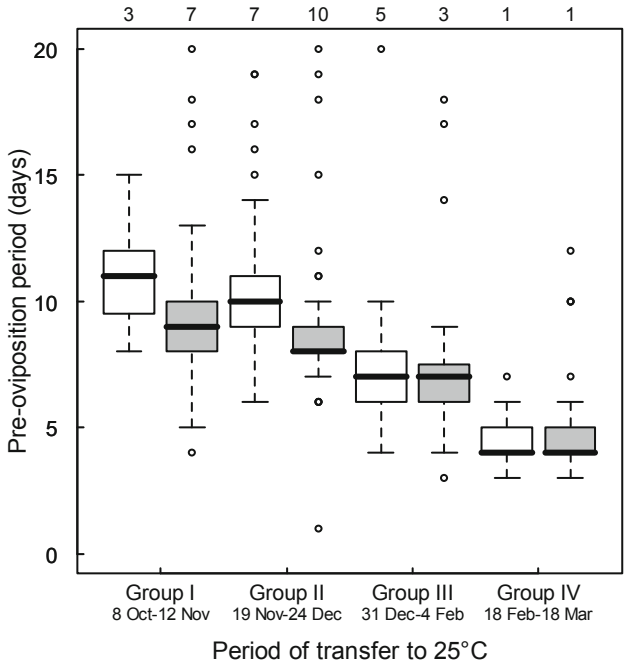

Figure 3 *Distribution of pre-oviposition periods of* Harmonia axyridis *females after transfer from outdoor hibernation cages to conditions representing short-day (25°C, 12:12 L:D) (white box) and long-day (25°C, 12:12 L:D) (grey box). The results are clustered in four groups with different dormancy lengths. The boxes represent the first to third quartile range with the thick line indicating the median. The whiskers represent a 95% confidence interval of the data. Outliers (censored and observed values) are represented by open circles; some points (16 (14 censored values) for short-day and 21 (19 censored values) for long-day) lie outside the plot range and are not shown. The number of outliers that is not shown in each group is given at the top of the graph.*

Discussion

As long as diapause has not terminated, it persists even when conditions temporarily improve. When diapause has ended, insects stay dormant in a state of post-diapause quiescence as long as unfavourable conditions are encountered, but as soon as favourable conditions arrive they will respond and resume development and reproduction (Tauber et al. 1986). The observed photoperiodic response (the difference in pre-oviposition period under short and long photoperiods) until mid-December indicates that *H. axyridis* starts overwintering in a state of diapause. By the end of December the pre-oviposition periods under both photoperiods are equally long, showing that diapause has ended, as it is not maintained by the short photoperiod anymore. We may, thus, conclude that *H. axyridis* exhibits a short period of diapause.

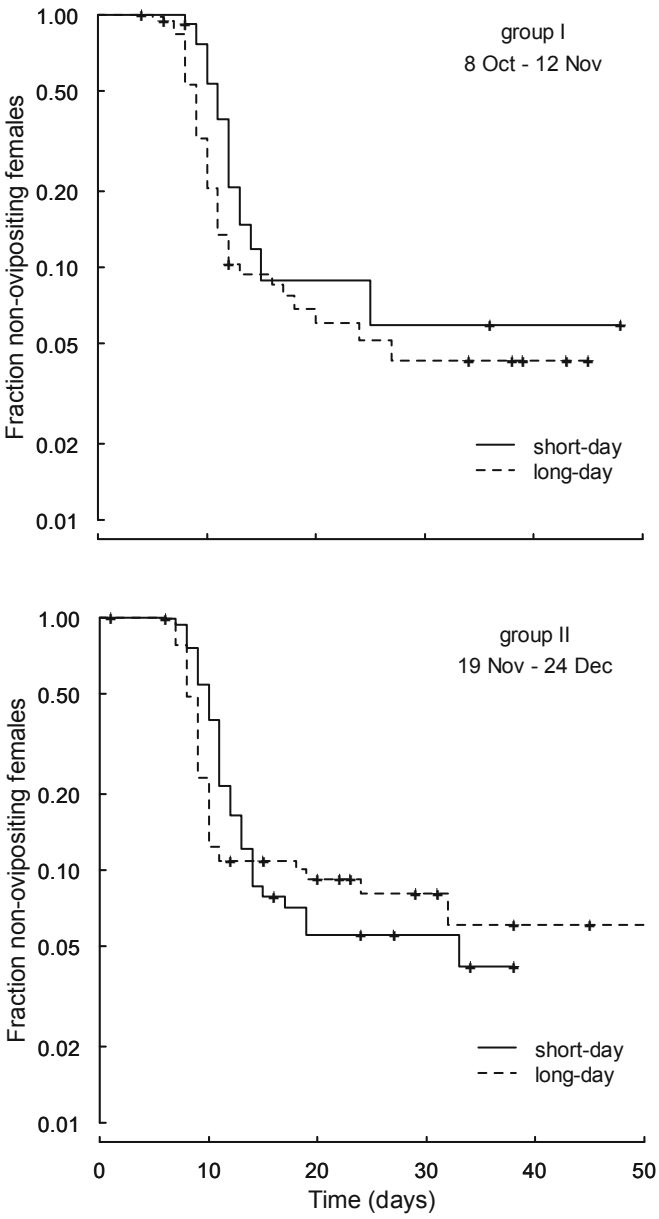

Figure 4 *Fraction of non-ovipositing females of* Harmonia axyridis *over time after transfer from outdoor hibernation cages to conditions representing long-day (25°C, 16:8 L:D) and short-day (25°C, 12:12 L:D). Observation of a female ended when the female died or when the first egg cluster hatched. The data are grouped on the basis of the length of the hibernation period of the beetles (see text). Censored values (females dying before oviposition) are marked with +.*

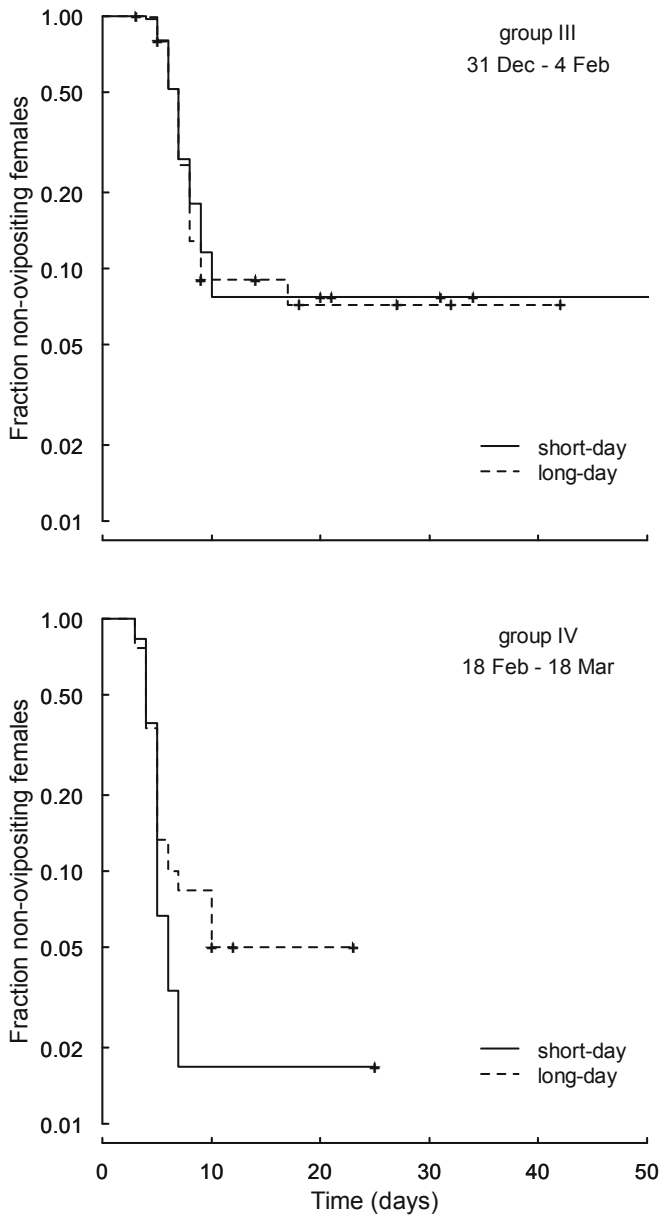

Figure 4 *Continued*

We suggested earlier that *H. axyridis* shifts from diapause to a post-diapause quiescent state in December (chapter 2). The current study confirms this, as until mid-December significantly longer pre-oviposition periods occur under short-day than under long-day conditions. Our hypothesis that *H. axyridis* starts overwintering with a period of diapause is further supported by the fact that *H. axyridis* also shows behaviour (migration and overwintering site selection before winter arrives) and physiological adaptations (regressed ovaries and a reduced fat body) that are typical of diapause (e.g. Obata 1986a; Nalepa et al. 1996; Iperti and Bèrtand 2001).

The native ladybirds *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) and *Adalia bipunctata* L. (Coleoptera: Coccinellidae) hibernate in diapause as well. In northern and western Europe *C. septempunctata* is univoltine and diapause occurs in every generation, while *A. bipunctata* is multivoltine, like *H. axyridis. Coccinella septempunctata* enters diapause in August/September and *A. bipunctata* in October. In December both species still show a response to photoperiod (Hodek et al. 1977; Obrycki et al. 1983; Brakefield 1985; Honek 1989; Hodek and Honek 1996; Brown et al. 2008a; Hodek 2012a). In March, the photoperiodic response of *A. bipunctata* is almost absent (Obrycki et al. 1983) and the beetles start emerging (Hemptinne and Naisse 1988). The diapause development in *C. septempunctata* is completed in mid-winter, but emergence from hibernation sites occurs only from March to May (Shands et al. 1972; Honek 1989; Nedved 1993). The total length of the diapause period of *H. axyridis* is shorter than that of *A. bipunctata* and much shorter than that of *C. septempunctata*.

Beetles of the species *C. septempunctata* and *A. bipunctata* show a stronger photoperiodic response than *H. axyridis* adults, indicating that diapause of the former two species is stronger. For beetles sampled in October the difference in pre-oviposition periods under long and short photoperiod is about fifty days for *C. septempunctata* and more than sixty days for *A. bipunctata*, while more than half of the females of both species do not start oviposition at all under short photoperiods (Hodek and Ruzicka 1979; Obrycki et al. 1983). Instead, for the October sample in this study with *H. axyridis* (group I: October–half November) the difference between the light regimes in median pre-oviposition period was two days, and no more than 5% of the females did not oviposit at all under short-day conditions. This shows that diapause of *H. axyridis* is weaker than that of both native species.

In this study, the median pre-oviposition periods for the long-day groups II, III, and IV (median (ci): 8 (7.7–8.3), 7 (6.6–7.4), and 4 (3.7–4.3) days), showed a similar trend as those found in the previous study (median (ci): 10 (9.4–10.6), 8 (7.6–8.3), and 5 (4.7–5.3) days respectively) (chapter 2), but they were shorter. In the winter of 1992–1993 Iperti and Bèrtand (2001) found median pre-oviposition periods in *H. axyridis* that were twice as long as those in this study. This was shortly after the first field experiments were conducted in south-eastern France in 1990 (Brown et al. 2008a). We therefore expect that the beetles had not yet adapted to the local conditions. Furthermore, environmental conditions (long-term averages) are comparable for south-eastern France and the Netherlands during the period of migratory flights and the first period of diapause (chapter 2). Although we cannot exclude the potential role of the use of an artificial diet versus natural prey in the difference between these and

our present results (Hodek and Honek 1996), this observation suggests that pre-oviposition period has shortened over the last two decades.

The apparent shortening of pre-oviposition period over the last decade may indicate a rapid change in diapausing behaviour of the *H. axyridis* population in Europe. This is supported by the fact that Berkvens et al. (2010a) were not able to induce diapause anymore in a field-collected population in 2008, while they still managed to do so in 2005 with the fourth generation of a field-collected population (Berkvens et al. 2008b).

When a population enters a new area, new and interesting changes in diapause behaviour can occur while the species adapts to the new circumstances. The Colorado potato beetle (*Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae)), for example, has shifted from diapause induced by low food quality in its native range (southern Mexico), to diapause induced by short photoperiod in the new area (northern USA and Europe), with the critical photoperiod depending on the latitude. Its behaviour has changed, too. In the new area it buries itself in the ground during diapause to avoid freezing temperatures, while it stays above ground in its native area (De Wilde and Hsiao 1981; Hsiao 1985).

Compared with the native species *C. septempunctata* and *A. bipunctata*, exotic *H. axyridis* has a short and remarkably weak diapause, which apparently does not reduce overwintering survival (chapter 4). On the other hand, this short diapause is not directly advantageous for *H. axyridis* either, as the moment that adults become active in spring is similar for all three species (chapter 2), and thus population build-up will generally start at approximately the same time. However, our data seem to suggest that, since diapause in *H. axyridis* lasts only a short period of time and appears to be weaker than that of native species, *H. axyridis* might be able to respond more opportunistically to variation in winter-climatic regimes. We hypothesise that this flexibility may give *H. axyridis* an advantage over native competitors, especially under present trends of climate-change, with earlier onset of spring conditions in western Europe (Hodkinson 2011). However, considerable testing is needed to confirm our hypothesis, which may be further explored by simultaneously monitoring hibernation, overwintering survival, and spring activity of *H. axyridis* and native species at the same location(s). Thus, it probably is not the *mode* of hibernation but the plasticity and adaptability of its diapausing behaviour that contributes to the invasion success of *H. axyridis*.

Acknowledgements

We would like to thank the following persons and organisations for various contributions: SW Benedictus-van den Berg, CAJM van Boxel, Mr & Mrs Van den Berg-van Boxel, I Folkertsma, A. Hakman, Mr & Mrs Huijsman, Mr & Mrs Van der Kamp, J van Kemenade, K Kots, B Lodeweges, AJM Loomans, G Mekking, MJ Raak, D Rozeboom, A Siepel, J Spitzen, JM Stam, T Timmer, W van Tol, E Voorhout, and PS van Wielink.

4

Winter survival of *Harmonia axyridis* in the Netherlands

BIOLOGICAL CONTROL (2012) 60: 68-76

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Abstract

Since the establishment of *Harmonia axyridis* in Europe, populations of native ladybirds have decreased. Overwintering survival is one of the aspects of the biology of *H. axyridis* that may contribute to its firm establishment and invasion into a new area. In this study winter survival of five wild *H. axyridis* populations was assessed under natural and semi-natural conditions, with a focus on the potential influence of location and orientation on winter survival.

Overwintering survival of *H. axyridis* in the Netherlands is high: 70.8% to 88.2%. When overwintering at one central site, populations sampled at five locations showed statistically significant different mortality rates. Furthermore, winter survival of *H. axyridis* at the sample sites was higher when beetles were hibernating at the southwestern sides of buildings, where most aggregations of ladybirds were found. Survival was higher at sheltered sites compared to exposed sites.

Harmonia axyridis has a comparable or higher overwintering survival than most common native ladybird species. A high overwintering survival results in a large post-hibernation population in spring, leading to a rapid population build-up. Thus, the high winter survival probably contributes to the success of the exotic *H. axyridis*.

Introduction

The predatory multicoloured Asian ladybird, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), is indigenous to Asia. It has several characteristics that make it useful for biological control of aphid pests: it has a high voracity and dispersal capacity and a multivoltine life-cycle (Koch 2003; Brown et al. 2008a). After numerous releases for biological control purposes in the USA as of 1916, the first established population was recorded there in 1988 (Koch 2003; Brown et al. 2008a). Since then, it has spread through most of the USA, as well as Canada (Koch et al. 2006a). After introduction in Europe in 1982, it took until 2000–2001 before *H. axyridis* had established in Europe. Since then it has spread rapidly across the continent and has currently established in 26 European countries (Brown et al. 2008a; 2011b). In autumn 2002 it was first recorded in the field in the Netherlands (Cuppen et al. 2004a), and by 2006 it had spread throughout the country (Brown et al. 2008a).

Besides characteristics favourable for biological control, *H. axyridis* has a low host specificity, consuming a wide range of aphid species as well as other soft-bodied insects, pollen, nectar, and larvae and eggs of several coccinellid species, including its own (Tedders and Schaefer 1994; Koch 2003; Pervez and Omkar 2006; Pell et al. 2008; Lundgren 2009). Additional characteristics that *H. axyridis* has in common with other high-risk species are: large size (Ware and Majerus 2008), low habitat specificity (Brown et al. 2008a), high fecundity (e.g. Hukusima and Kamei 1970; Stathas et al. 2001), low larval mortality (Hukusima and Ohwaki 1972; Dmitriew et al. 2009), and an aggressive nature (Yasuda et al. 2001; Michaud 2002b; Yasuda et al. 2004).

Insects with this combination of characteristics constitute a risk in that they can become an invasive species. Van Lenteren et al. (2003; 2006; 2008) showed that of 31 biological control agents tested, *H. axyridis* scored second highest on the list of most risky, exotic, natural enemies. Indeed, *H. axyridis* is regarded as an invasive species: it is still expanding its range, and its numbers increase while populations of native ladybirds decrease (Colunga-Garcia and Gage 1998; Michaud 2002b; Alyokhin and Sewell 2004; Harmon et al. 2007; Finlayson et al. 2008; Adriaens et al. 2010; Brown et al. 2011a).

Several aspects of the overwintering biology of *H. axyridis* can contribute to its establishment in and invasion of a new area. In earlier work, we evaluated the mode of overwintering of *H. axyridis* in Europe and the moment it becomes active in early spring. Both aspects were deemed unlikely to have promoted its invasiveness in Europe (chapter 2 and 3). Another aspect which might play a role is winter survival. If *H. axyridis* has a higher winter survival than other coccinellid species, it will start with a relatively large population in spring, which contributes to a rapid population build-up.

Overwintering of *H. axyridis* starts in autumn on warm, calm days that follow a colder period. In search of suitable hibernation sites beetles fly in large swarms towards conspicuous silhouettes on the horizon (e.g. rocks, trees, buildings, or poles). After landing, the beetles walk around, searching for cracks and crevices where they can overwinter, and form aggregations of up to a thousand beetles. In spring, the beetles migrate from their hibernation sites toward their feeding grounds (Tanigishi 1976; Obata 1986b; Sakurai et al. 1993; Kidd et al. 1995; Nalepa et al. 2005; Wang et al. 2011). Only limited published data are available on overwintering survival of

H. axyridis in Europe. Winter survival of *H. axyridis* overwintering outside was high (≥ 60%) in south-eastern France (Iperti and Bèrtand 2001), northern Italy (Bazzocchi et al. 2004), and Japan (Watanabe 2002), while winter survival of wild *H. axyridis* overwintering inside as well as in leaf litter in Quebec, Canada was low (≤ 10%) (McClure 1987; Labrie et al. 2008).

Insight in overwintering survival of *H. axyridis* will help to explain the invasive nature of this species and contribute to a more general comprehension of the dangers of introduced biota. This, in turn, can pinpoint factors that need to be investigated for the safe introduction of exotic species as biological control agents. In this study, the survival of wild *H. axyridis* populations under semi-natural conditions was examined and factors affecting the overwintering success of *H. axyridis* were determined.

Materials and methods

Collecting beetles

Survival of adult *H. axyridis* beetles was studied in the Netherlands during the winter of 2009–2010. In the second half of November 2009 beetles were collected from hibernation aggregations at five locations: Alkmaar, Houten, Loenen, Kootwijk, and Zundert (first five in table 1).

Survival of each population was monitored at two sites: at the sampling site itself and at a central test site (N 51 59 32, E 5 39 43). The latter is an experimental farm of Wageningen University. Beetles were transported to Wageningen at ambient

Table 1 *Sampling sites, coordinates, dates, description, and sample size of hibernating* Harmonia axyridis *in 2009. N = number of beetles used.*

Site	Coordinates	Date	Description location and sampling site	N
Alkmaar	N 52 37 57, E 4 44 35	19 Nov 2009	Urban area, inside unheated church tower on stone wall	1498
Houten	N 52 1 39, E 5 9 38	27 Nov 2009	Urban area, inside unheated church tower on stone wall	1544
Loenen	N 52 6 2, E 6 0 23	17 Nov 2009	Woodland area, outside, between stone wall and shutters	763
Kootwijk	N 52 10 39, E 5 45 39	13 Nov 2009	Woodland area, outside, between stone wall and shutters	2125
Zundert	N 51 30 38, E 4 38 36	26 Nov 2009	Woodland area, outside, between stone wall and shutters	1609
Alphen	N 52 8 33, E 4 39 22	8 Dec 2009	Urban area, outside, in steel window frames	99
Deelen	N 52 3 53, E 5 53 23	25 Nov 2009	Rural area, outside, between stone wall and shutters	622
Engelbert	N 53 12 31, E 6 38 50	21 Nov 2009	Rural area, inside unheated church on stone wall	65
Tholen	N 51 31 55, E 4 13 8	26 Nov 2009	Urban area, in unheated church tower on stone wall	555

temperature (7–13°C) in 1 litre jars with wrinkled filter paper, covered with netting. Before the beetles were put into monitoring cages at the central test site, they were kept in an unheated shed (3–13°C).

Winter survival experiment

Design of overwintering sites

To monitor overwintering, the beetles were put into gauze bags (10 x 50 cm, gauze diameter 1 x 1 mm) with a metal spiralling frame (3 x 6 x 40 cm) to allow free movement within the bags. Since the beetles were able to hide in the folds of the bags, as they are used to doing in cracks and crevices, and since the gauze diameter was fairly large to allow for air circulation, we assume that the bags did not affect either overwintering behaviour or microclimate to a great extent. Moreover, if bags had a slight effect on mortality, this would still not explain any differences between locations or orientations as all bags used were identical. Each bag contained approximately 100 beetles. For the small Loenen population each bag contained 50 individuals.

Winter survival at sampling sites

At each sampling site, survival was monitored at two orientations: south, where most beetles were found, and the opposite, north. Temperature was recorded with a datalogger (Gemini Tinytag TPG 1500 & TGU 1500) at the orientation where most beetles were found.

Four bags with beetles were placed at each of the two orientations: in Alkmaar and Houten on the inside of the outer stone walls, and in Kootwijk, Loenen, and Zundert outside behind wooden shutters on the stone wall. In Kootwijk, another four bags were placed in leaf litter under a hedge. At the central test site, four additional bags, containing beetles from Kootwijk, were placed in leaf litter under shrubs.

Winter survival at the central test site

Winter survival at the central test site was monitored in outdoor cages behind six wooden shutters on the southern wall of the experimental farm. This setup has been described in chapter 2. In the current experiment, bags with beetles were transferred to the outdoor cages on 4 December 2009. They were suspended from hooks in the upper bar of the outer frame. Due to the position of heaters and a cooling chamber inside the building, the temperature behind the three left-hand shutters was on average 3.3°C higher than behind the three right-hand shutters during winter 2008–2009 (chapter 2). Five bags were randomly placed side by side behind every shutter, each bag containing beetles from another sampling site. Behind shutters 2 and 5, temperature was recorded every 30 min (MicrologPRO, Fourier Systems).

Monitoring survival

The number of surviving beetles was determined at regular intervals: every four weeks at the sampling sites and every two weeks at the central test site. Beetles that walked around, that showed any movement of antennae or legs, or were reflex-bleeding, were considered alive and were allowed to continue hibernation. Dead beetles were removed. Colour morph and sex of dead individuals were recorded (McCornack et al.

2007). Three colour morphs were distinguished: f. s*uccinea*, f. *spectabilis,* and f. *conspicua* (Komai 1956; Michie et al. 2010). To reduce disturbance of the beetles by temperature change, survival was determined outside, at the sampling site itself, or, at the central test site, in a climate room at 4°C.

End of overwintering
At the end of winter, during warm periods, large numbers of beetles were observed in the field, indicating that *H. axyridis* had left its overwintering sites. The activity of overwintering *H. axyridis* placed in field cages in half and full shade confirmed this. Based on these observations, monitoring was terminated to avoid registration of artificial mortality caused by increased post-hibernation activity at higher temperatures while beetles were still caged.

On 18 March 2010 the experiment was terminated at the central test site and between 22 March 2010 and 24 March 2010 it was terminated at the sampling sites. All bags containing beetles were transported to Wageningen. Surviving beetles were counted and colour morph was recorded on the day the experiment was terminated. Later, beetles from the central test site were sexed. Beetles walking around after 24 h at ambient temperature were considered alive. Beetles that did not move were considered dead in further analysis (Labrie et al. 2008).

Survival at constant temperature
One extra sample of beetles from each location, each in a separate bag, was kept at constant temperature (5°C, L:D 0:24, RH not measured) from 4 December onwards. Survival was checked every fortnight. The experiment continued until all beetles had died as no spring conditions arrived that activated the ladybirds as in the monitoring described above. The median survival time ($LTime_{50}$) was determined.

Orientation
During sampling, the distribution of the overwintering aggregations over the compass orientations was recorded at the sampling sites and at four additional locations: Alphen aan den Rijn, Deelen, Engelbert, and Tholen (table 1). For Houten and Kootwijk distribution was determined on the basis of photographs of all aggregations found. Previous tests have shown that precise counting of photographs gives a good estimate of the aggregation size (Schellekens 2006).

Fat body content
In autumn 2008, beetles were collected in Kootwijk and Loenen. These beetles continued overwintering at the central test site and were frozen at regular intervals as described in chapter 2. Beetles frozen on 22 December 2008, 2 February 2009, and 3 March 2009 were dissected, and the fat body content was determined. In the winter of 2009–2010, fat body content was determined in dissected beetles. This sample included the whole range from well-fed specimens in late autumn to starved individuals in early spring and provided a good basis for the scale we used in this study. The scale ranged from undeveloped fat body (0, no fat) to well-developed fat body (5, very fat).

Statistical analysis

Survival analysis analyses the time until a certain event occurs, here the death of an individual overwintering ladybird. It also includes censored data: situations where the experiment is ended before the event has occurred. Data were analysed with Cox's proportional hazards model as described in chapter 2. Kaplan-Meier's product limit estimator was used to plot the data and the Log-rank test was used to test whether non-proportional covariates had a significant effect on the mortality patterns. If so, the model was stratified for this. Data were analysed using PASW Statistics (18.0.3, 9 Sept 2010).

The distribution of the aggregations over eight orientations was tested using a χ^2-test to detect deviations from a discrete uniform distribution. Whether the fat body scales differed at the three sample dates was tested with Fischer's exact test (SPSS 15.0.1.1, July 2007).

Results

Winter survival

Winter survival at sampling sites

The average survival at the sampling sites in winter was 88.2% (SE 5.45) (figure 1). Survival differed significantly between almost all sites (pairwise Log–rank tests with Bonferroni correction (confidence level 0.05/10), $P \ll 0.001$), except for Loenen, where survival did not differ from Alkmaar ($\chi^2_1 = 3.08$; $P = 0.079$), Kootwijk ($\chi^2_1 = 2.05$; $P = 0.153$), and Zundert ($\chi^2_1 = 5.09$; $P = 0.024$). In Alkmaar and Houten the beetles hibernated inside the building while in Kootwijk, Loenen, and Zundert the beetles hibernated outside the building, behind shutters. We considered type of shelter and orientation as separate covariates and found that the effects are independent. Overwintering at a north-eastern orientation resulted in a doubling (effect = 1.99) of the mortality rate compared to overwintering at the southwestern orientation, whereas overwintering outside resulted in a 5.35 times higher mortality rate compared to overwintering inside. Therefore, the ratios of mortality rates 'south-inside':'south-outside':'north-inside':'north-outside' are approximately 1:2:5:10 (Cox' regression model, Wald test = 125.05, df = 3, $P \ll 0.001$). Still, mortality was low in all cases as it did not exceed 11.8%. Including the covariate colour morph in this analysis did not influence the mortality rate significantly.

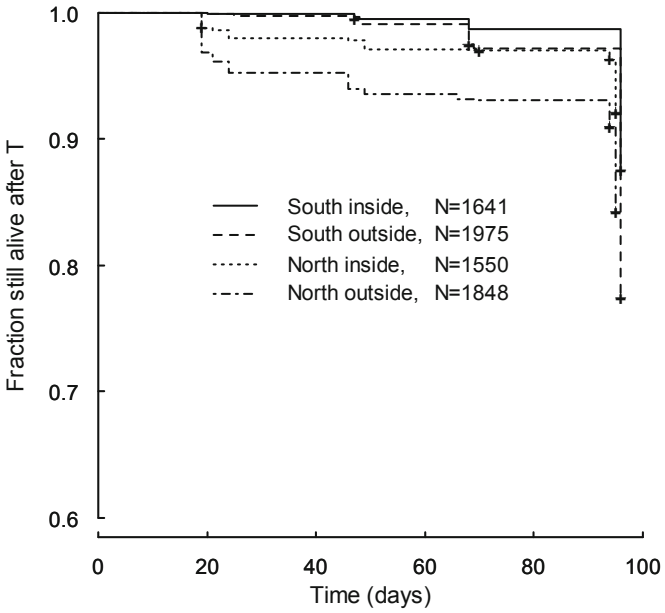

Figure 1 *Winter survival of* Harmonia axyridis *adults at five sampling sites in the Netherlands. The data are grouped by shelter (inside or outside) and orientation (south-west or north-east), see text. N = number of beetles used.*

Winter survival at central test site

Average winter survival at the central test site was 70.8% (SE 10.7) (figure 2). The average temperature behind the three shutters on the left (6.3°C; range –3.9°C to 20.8°C) was 3.9°C higher than on the right (2.4°C; range –5.8°C to 17.2°C), being 0.6°C higher than in 2008–2009 (chapter 2). Beetles behind the shutters on the left had a shorter mean survival time than those behind the three shutters on the right (Log–rank test, $\chi^2_1 = 521.8$, $P \ll 0.001$). Therefore, the data were stratified for the left/right side of the shutters in further analysis.

The origin of the population influenced survival. In a model stratified for left/right shutters, beetles behind the left shutters from woodland (Kootwijk, Zundert, Loenen) had a longer mean survival time than beetles from an urban area (Alkmaar, Houten) (for all significant pairwise comparisons: Log-rank tests with Bonferroni correction (confidence level 0.05/10), $\chi^2_1 > 53.8$, $P \ll 0.001$). Behind the right shutters all beetles showed similar survival, except for beetles from Kootwijk, which had a lower survival than beetles from Alkmaar, Houten, and Loenen (pairwise Log-rank tests with Bonferroni correction, $\chi^2_1 > 8.83$, $P < 0.003$). Neither colour morph nor sex influenced winter survival significantly.

Until 1 March 2010 survival remained above 90% (SE 3.11), but on 15 March 2010 average survival was 79.7% (SE 7.5). Thus, survival dropped rapidly at the end of winter.

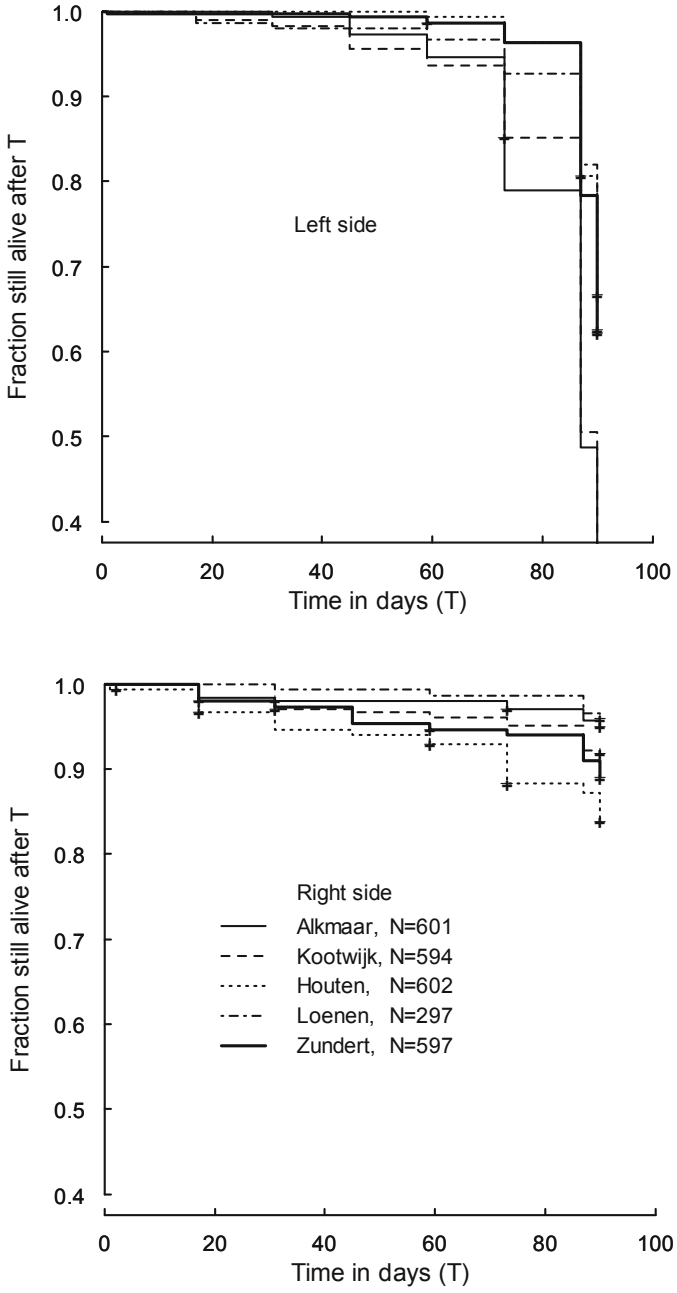

Figure 2 *Winter survival of* Harmonia axyridis *adults, originating from five sites in the Netherlands, at the central test site. N = total number of beetles used. Average temperature on the left was 6.3°C (range –3.9 to 20.8°C) and on the right was 2.4°C (range –5.8 to 17.2°C).*

Survival in leaf litter

Survival of beetles overwintering in leaf litter was low with relatively high mortality from the start of winter onwards. In Kootwijk, 8.2% survived and at the central test site 12%. In Kootwijk mean survival time of the beetles in leaf litter was lower than that of beetles overwintering at the north-eastern and southwestern sites (Log-rank test, $\chi^2_1 > 491.6$, $P \ll 0.001$). At the central test site, mean survival time of the beetles in leaf litter was lower than that of the beetles from the Kootwijk population overwintering behind shutters (Log-rank test, $\chi^2_1 > 302.9$, $P \ll 0.001$).

Survival at constant temperature

On 18 March, at the end of the winter period, survival at a constant 5°C temperature was on average 95.1% (range 90.6%–99.0%). The median survival time ($Ltime_{50}$) was 171 days (range 144–185 days), which means that 50% of the beetles survived until 7 June. At 28 September (about 11 months after the collection of the beetles) all beetles had died.

Orientation

The ladybird clusters found in the field were not randomly distributed over all orientations ($\chi^2_7 > 455$, $P \ll 0.001$). Most beetles were found at the orientations south, south-west, and west, except for Loenen and Kootwijk (figure 3).

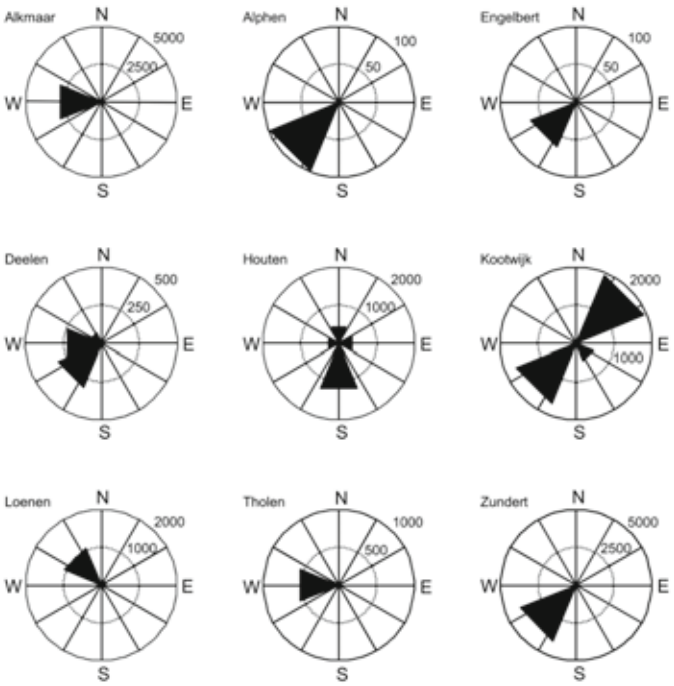

Figure 3 *Distribution of hibernating* H. axyridis *adults over the compass orientations at nine sites in the Netherlands. Numbers at concentric circles indicate scale (number of beetles).*

Fat body content

Fat body content was determined for 127 adults and differed significantly between the three sampling dates (Fischer's exact test, P = 0.039). The proportion of 'very fat' and 'fat' beetles decreased during the winter while the proportion of 'low fat' and 'very low fat' beetles increased. Beetles with 'no fat' were only observed in March (figure 4).

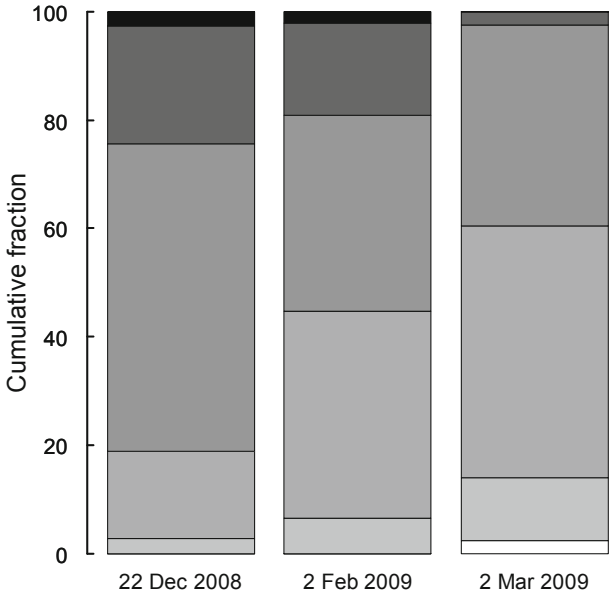

Figure 4 *Fat body content of overwintering* Harmonia axyridis *adults over time. Fat body content is determined at the three sampling dates and categorised using six different shades of grey, from no fat (white) to very fat (black).*

Discussion

Overwintering survival of *H. axyridis* in the Netherlands is high: 88.2% at the sampling sites and 70.8% at the central test site. The difference in survival between the sampling sites and the central test site might be attributed to handling and disturbance of the beetles during transport to the central test site and the more frequent counting at that site. In addition, the central test site might be suboptimal for overwintering as the beetles did not select the site themselves. Survival also differed between the populations overwintering at the five sampling sites; it was highest at Houten and lowest at Zundert. The survival percentages we found are in range with those of previous studies in Italy (59–100%), France (> 91%), and Japan (90% and 46–94%) (Tanigishi 1976; Iperti and Bèrtand 2001; Watanabe 2002; Bazzocchi et al. 2004). Apparently, *H. axyridis* is very well able to survive European winters outside. In Canada, where winters are generally much colder than in Europe, no beetles survived outside buildings, and inside buildings, being 'cold-free spaces', only 24.8% survived (Labrie et al. 2008).

Most ladybird clusters were found at the orientations south, south-west, and west. As the survival data show, survival of *H. axyridis* was higher at the southwestern sides of the buildings, where, in general, most beetles were found. Survival was also higher at the sheltered sites. After landing on a building, migrating beetles search for cracks and crevices to hide (Tanigishi 1976; Sakurai et al. 1993; Nalepa et al. 2004; Nalepa 2007; Wang et al. 2011). Apparently, natural selection has resulted in a preference for southwestern and sheltered spots, where survival is higher. We assume that sheltered locations provide more constant conditions with less fluctuation in temperature, humidity, and wind. It is likely that southwestern locations are also somewhat warmer and drier, which probably causes fewer (fungal) infections and diseases. Brakefield (1985) also reported higher mortality of *Adalia bipunctata* L. (Coleoptera: Coccinellidae) at exposed sites, suggesting that higher air humidity results in wet body surfaces, which makes the insect freeze internally at higher temperatures.

Only at two locations (Loenen and Kootwijk) we did not find all beetles at the southwestern side, which might have been caused by the landmarks being partly hidden by trees. During migration to overwintering sites, *H. axyridis* first orients to macro sites: large visual landmarks that form silhouettes against the horizon (Tanigishi 1976; Obata et al. 1986b; Nalepa et al. 2005; Wang et al. 2011). To allow orientation towards a landmark, that landmark needs to be visible from a distance. Both in Loenen and in Kootwijk the buildings on which the beetles landed were surrounded by trees. In Loenen only the northwestern side of the building was exposed, while in Kootwijk the southwestern and north-eastern sides were exposed. And, indeed, most beetles are found at those orientations. When beetles land at a sub-optimal side, they do not, apparently, walk any further in search of the optimal orientation.

Adalia bipunctata prefers the east- to south-west-facing side of poplar trees (1985); *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) prefers hibernacula on slopes that face south-east to south-west (Honek 1989) in the Czech Republic and mounds of pampas grass that face south and east in Japan (Takahashi 1993). However in these studies, orientation preference has not been linked with winter survival.

The differences in survival between left and right shutters at the central test site can best be explained by the difference in temperature, which was on average 3.9°C higher on the left (6.3°C, range –3.9 to 20.8°C) than on the right (2.4°C, range –5.8 to 17.2°C) in winter 2009–2010. At the sampling sites average temperatures were presumably lower on the north side than on the south-west side and higher at the sheltered locations. Differences in survival at the sampling sites, however, cannot be explained by the measured temperatures alone as those do not point unambiguously in the same direction. Possibly the relationship between temperature and survival is non-linear, as previous studies on survival of *H. axyridis* at constant temperature indicate. In Japan (Watanabe 2002), China (Ma et al. 1997), and Canada (Berthiaume et al. 2003) survival was highest at 0°C, closely followed by 5°C, and then by –5°C. In Belgium survival was also higher at 0°C (20–60%) than at –5°C (0–57%) (Berkvens et al. 2010a; N Berkvens pers. comm.), while in Canada Labrie et al. (2008) found 45% survival at –5°C over a period of 4 months. At more extreme temperatures, survival drops further: studies report no survival for more than 10 weeks at –7.1°C (Ma et al.

1997), no survival for more than 12 weeks at –10°C (Berthiaume et al. 2003), no survival for more than three months at +10°C (Labrie et al. 2008).

The decrease in survival at higher temperatures can be explained by desiccation or, as beetles are more active, by a depletion of energy reserves (Watanabe 2002). Labrie et al. (2008) showed that *H. axyridis* contained less lipids at 10°C than at –5°C, which indicates a higher use of energy reserves at 10°C. Sakurai et al. (1992) reported decreasing fresh weight over winter and a decreasing fat body from February onwards. Two other studies also found a decreasing fat body over winter (Iperti and Bèrtand 2001; Zhao et al. 2008). Our study, too, confirmed this.

Mortality at temperatures lower than the optimal survival temperature can be attributed to chilling injury or freezing. If freeze-intolerant species, such as *H. axyridis*, cool down below their super cooling point (SCP), they freeze and die. During this study the lowest temperatures measured were –5.8°C behind the shutters at the central test site and –10.9°C at the sampling sites. These values are well above the SCPs measured throughout winter for *H. axyridis*: –18.2 to –14.0°C (Berkvens et al. 2010a), –18°C (Watanabe 2002), –18.1 to –16.9 (Zhao et al. 2008), and –22.5 to –12.3°C (Koch et al. 2004) and also well above median lethal temperatures (Ltemp50) for *H. axyridis* measured in mid-winter: –16.8 to –14.1°C (Berkvens et al. 2010a), –16°C (Watanabe 2002), and –17.5 to –12.5°C (Koch et al. 2004). This indicates that mortality in this study was probably not caused by freezing but rather by chill injury, which is pre-freeze mortality due to cumulative effects of cold stress after being exposed to low temperatures over a long period of time (Bale 1993; Watanabe 2002; Danks 2005; Berkvens et al. 2010a). Chill-tolerant species are able to survive at low temperatures normally encountered during winter but show non-freeze mortality above SCP due to chill injury. *Harmonia axyridis* can be regarded as a chill-tolerant species that shows a low degree of pre-freeze mortality (Bale 1993).

For chill-tolerant and chill-susceptible species, the $Ltime_{50}$, reflecting the cumulative effects of cold stress, seems a more reliable predictor of field survival than the SCP since it better represents the effects of temperature experienced during temperate winters (Hatherly et al. 2005; Berkvens et al. 2010a). This assumption is supported by the fact that the SCP did not adequately explain the distribution of *H. axyridis* in the north of the United States (Koch et al. 2004). We found an $Ltime_{50}$ of 171 days, which, when corrected for the 40-day-late start after migration, is in line with other studies (Watanabe 2002; Berthiaume et al. 2003; Galvan et al. 2008) and indicates a high winter survival capacity for *H. axyridis*.

At the end of the overwintering period, survival dropped rapidly, as also found by Watanabe (2002). In our study, this sudden increase in mortality is probably caused by high post-hibernation activity while the beetles were still caged and not able to move away from high-temperature sites as they would do in the field. Termination of the experiment was determined by the activity of beetles in field cages and by the observation of large numbers of active beetles outside. However, under field conditions the beetles presumably leave a spot during short spells of higher temperatures, e.g. due to sunshine, and search for shelter when temperature drops again. In the experimental setup we could not mimic this situation fully, supposedly

causing higher mortality. We did not observe any differences in the moment of increased activity in the field compared to the experiment.

Another factor influencing winter survival is the quality of the beetles: low quality individuals, that have not developed sufficient fat reserves, have a lower chance of surviving winter (Honek 1997).

A third factor influencing winter survival is infection with parasites and pathogens. Especially infection with hypocrealean fungi (Ascomycota) and with the mite *Coccipolipus hippodamiae* McDaniel and Morrill (Podapolipidae) were found to influence winter mortality (Roy et al. 2011b). Steenberg and Harding (2009a) found hypocrealean fungi in wild *H. axyridis* adults in Denmark (4.5%) and state that fungal prevalence reaches levels comparable to those published for coccinellid species native to Europe. However, laboratory studies showed that *H. axyridis* is less susceptible to *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Cordycipitaceae) than native species (Cottrell and Shapiro-Ilan 2008; Roy et al. 2008). For the native species *A. bipunctata* and *C. septempunctata*, *B. bassiana* caused mortality rates between 0% and 95% (Lipa et al. 1975; Mills 1981a; Hemptinne 1988).

Adults of *H. axyridis* infected by the mite *C. hippodamiae* were found in Poland at low rate (3.7%), but it is unclear whether the mite population has permanently established in *H. axyridis* populations or only incidentally affects this beetle (Rhule et al. 2010). The infection rate is low compared to that of *A. bipunctata* (44.9%) and *A. decempunctata* (10.8%) and is similar to that of *Calvia quartordecimguttata* L. (Coleoptera: Coccinellidae) (4.2%) (Webberley et al. 2004).

The ectoparasitic fungus *Hesperomyces virescens* (Laboulbeniales; Ascomycota) infects *H. axyridis* in the USA (e.g. Riddick and Schaefer 2005) and Germany (Steenberg and Harding 2010b), but Laboulbeniales are thought to do little harm to their hosts (Roy et al. 2011b). However, at relatively high (8°C), constant overwintering temperature, it potentially reduces survival of *H. axyridis* males (Riddick 2010). Recently, infection of *H. axyridis* with nematodes belonging to *Parasitylenchus* sp. (Allantonematidae; Tylenchomorpha) was found in Denmark (Harding et al. 2011) and the USA (Roy et al. 2011b). This nematode is not lethal but does affect reproduction (Ceryngier and Hodek 1996).

Frequent and intergenerational mating are important factors enhancing the transmission of mites (Webberley et al. 2004) and *H. virescens* (Riddick and Cottrell 2010). As *H. axyridis* is multivoltine, overwinters in aggregations, and mates before leaving the hibernation sites, we suppose that in the future, mite and *H. virescens* infection can expand and influence winter survival.

In our winter 2009–2010 population, the prevalence of pathogens and parasites was rather low (CL Raak-van den Berg pers. obs.) and not a major factor influencing survival during this winter season.

Only 8–12% of the ladybirds overwintering in leaf litter survived. McClure (1987) also reported low survival of *H. axyridis* in leaf litter (0.2–10%), as did Labrie et al. (0%) (2008). Whereas leaf litter is a suitable habitat for overwintering of other ladybirds (e.g. *Coleomegilla maculata* De Geer (Coleoptera: Coccinellidae) (Labrie et al. 2008), *Adalia decempunctata* L. (Coleoptera: Coccinellidae), *C. septempunctata,* and *Propylea quatuordecimpunctata* L. (Coleoptera: Coccinellidae) (Majerus 1994; Hodek and Honek

1996)), it is not so for *H. axyridis*. Leaf litter offers a very different microclimate than the hibernacula that *H. axyridis* uses for overwintering. This probably results in an increased chance to become infected by fungal diseases, especially when *H. axyridis* is not able to avoid an area with living spores as *C. septempunctata* is (Ormond et al. 2011). Thus, when measuring winter survival, it is essential to mimic natural overwintering habitats of the species, in particular when evaluating the species as a potential biological control agent.

Compared to the most common Dutch native ladybird species *A. bipunctata* and *C. septempunctata*, *H. axyridis* has a comparable or higher overwintering survival (table 2). The ecological requirements for overwintering of *A. bipunctata* are similar to those of *H. axyridis,* as *A. bipunctata* also overwinters in buildings (Benham and Muggleton 1978) or under the bark of trees (Mills 1981a; Hemptinne 1985; Hodek and Honek 1996). An extension of this study, directly comparing survival of *A. bipunctata* and *H. axyridis* would have been preferred, if natural *A. bipunctata* would not have dropped dramatically (Adriaens et al. 2010; Brakefield and De Jong 2011; Brown et al. 2011a) in numbers, which made it impossible to conduct such experiments in the current study.

Table 2 *Outdoor winter survival rates (%) of* Harmonia axyridis, Coccinella septempunctata*, and* Adalia bipunctata

Harmonia axyridis	**Survival**	**Setup**
The Netherlands, this study	70.8	semi-natural, caged, wild
The Netherlands, this study	88.2	natural, caged, wild
Italy (Bazzocchi et al. 2004)	59–100	insectary, caged, reared
Japan (Watanabe 2002)	> 90	insectary, caged, wild
France (Iperti and Bèrtand 2001)	> 91	natural, caged, reared
Adalia bipunctata		
Italy (Bazzocchi et al. 2004)	52–100	insectary, caged, reared
Germany (Webberley and Hurst 2002)	93	semi-natural, caged, wild
The Netherlands (Brakefield 1985)	17–78	natural, not caged, wild
England (Majerus 1994)	76–79	natural, not caged
England (Majerus 1994)	70–73	semi-natural, caged
England (Mills 1981a)	64	natural, not caged, wild
Poland (Lipa et al. 1975)	52.3–96.4	natural, not caged, wild
Coccinella septempunctata		
USA (Cartwright et al. 1982)	46–59	semi-natural, caged, wild
USA (Shands et al. 1972)	6.3–80	semi-natural, caged, reared
Greece (Katsoyannos et al. 1997b)	40–80	insectary, caged, reared
Czech Republic (Honek 1997)	48	semi-natural, caged, wild
Czech Republic (Honek 1989)	30–40	natural, not caged, wild
England (Barron and Wilson 1998)	85–90	insectary, caged, wild
England (Zhou et al. 1995)	8–32	natural, caged, wild
England (Dean 1983)	33–77	natural, caged, wild
Poland (Lipa et al. (1975) according to Honek (1997))	67–81	natural, not caged, wild

The high overwintering survival of *H. axyridis* leads to a relatively large post-hibernation population in spring, leading to a rapid population build-up. We conclude that rather than the mode of overwintering (chapter 2), the high winter survival contributes to the invasion success of *H. axyridis*. We also suggest that it is important to determine overwintering survival of a polyphagous exotic species in comparison with the survival of indigenous species before introducing an exotic species as a biological control agent.

Acknowledgements

We thank the following persons for various forms of assistance during the project: Mr & Mrs Brandsma, AB Allema, SW Benedictus-van den Berg, CAJM van Boxel, K Helling, Mr & Mrs Huijsman, Mr & Mrs Van der Kamp, Mr & Mrs Klok, AJM Loomans, K Oku, G Mekking, MJ Raak, H van Roekel, D Rozeboom, H Schep, A Siepel, W van Tol, and E Voorhout.

4

5

Life histories of an invasive and native ladybird under field conditions during summer in a temperate climate

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Abstract

The multicoloured Asian ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) has been introduced in the USA and Europe as a biological control agent of aphids. Soon after its establishment negative effects on non-target organisms were reported and it is now regarded as an invasive species. Many characteristics of *H. axyridis* are thought to contribute to its invasion success, though most of these have been determined under laboratory conditions. Immature development time and immature survival of *H. axyridis* as well as of the native species *Adalia bipunctata* L. (Coleoptera: Coccinellidae) were measured in the field during summer in the Netherlands. Larvae were placed on lime trees in groups of either one or both species with ample food to determine if interspecific mortality occurred. Despite incomplete life tables, not unusual for field studies, development time and survival could be estimated for both species. Development time of both species is in line with data from laboratory tests under controlled conditions and immature survival can reach high levels (i.e. 44.4–100% for *H. axyridis* and 11.1–76.9% for *A. bipunctata*).

When sufficient prey is available, *A. bipunctata* and *H. axyridis* do not interact and intraguild predation does not seem to play a role in causing important mortality. The invasive *H. axyridis* has a considerably higher survival than the native *A. bipunctata* and, therefore, has a faster population growth, which is one of the factors that may explain its invasion success of *H. axyridis*.

Introduction

The multicoloured Asian ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) has been introduced in the USA and Europe as a biological control agent of aphids. The first introduction in Europe was in 1982, where it established in the late 1990s and has extended its range rapidly since 2002 (Brown et al. 2011b). Soon after its establishment negative effects on non-target insect species, fruit production, and human health have been reported, so that *H. axyridis* is now regarded as an invasive species (De Clercq and Bale 2011). The establishment of *H. axyridis* has been associated with a decline in native ladybird populations (see e.g. Alyokhin and Sewell 2004; Roy et al. 2012). Due to these negative effects, the ladybird is no longer commercially available in most of Europe (Van Lenteren 2012), except for France where a wingless mutant is still used (Biotop 2013).

Many characteristics of *H. axyridis* are thought to contribute to its invasion success. The species is polyphagous, eurytopic, multivoltine (Hodek et al. 2012), has a good dispersal ability (Brown et al. 2011b and references therein), a short generation time, a high immature survival, and a high fecundity (reviewed in chapter 8): all characteristics that contribute to a rapid population growth. Finally, *H. axyridis* is a strong intraguild predator (reviewed by Pell et al. 2008), benefitting from its high mobility, aggressiveness, large size (Yasuda et al. 2001), great ability to escape from attack by other ladybirds (Yasuda et al. 2001), and defence by chemical deterrence (Sloggett et al. 2011) and morphological structures (dorsal spines and anal disk) (Hautier et al. 2010; Osawa 2011).

Most biological knowledge of *H. axyridis* is based on laboratory studies, while we are interested in its field biology. In this chapter we address the following questions: 1) What is the immature development time and immature survival of the invasive *H. axyridis* and the native *Adalia bipunctata* L. (Coleoptera: Coccinellidae) under field conditions during summer in the Netherlands?; 2) Does co-occurrence of *H. axyridis* with *A. bipunctata*, under conditions of sufficient prey availability, result in intraguild predation or cause a different kind of mortality in one or both species?; and 3) Do the results of this experiment contribute to an explanation of the invasion success of *H. axyridis*? To answer these questions, we have estimated immature development and survival under field conditions. The ladybirds were placed in cages, so mortality due to natural enemies could be excluded. We have also quantified the effect of co-existence of *H. axyridis* and *A. bipunctata* on lime trees.

Material & Methods

Insects

Overwintering *H. axyridis* were field collected at hibernation locations and allowed to overwinter in outdoor cages or in a climate cabinet (5 ±1°C, 0:24 L:D) (chapters 2 and 4). *Adalia bipunctata* adults either originated from a laboratory culture of Leiden University, the Netherlands; were field collected at hibernation locations and overwintered similar to *H. axyridis*; or were field collected in spring shortly before the experiments.

Shortly before the experiments ladybirds were transferred to a climate chamber under warm conditions: 25±1°C (2009), 24±1°C (2010), 16:8 L:D. Pairs of males and

females were kept in Petri dishes (Ø 9 cm) lined with filter paper, received ample amounts of *Ephestia* eggs, bee pollen, and honey water, and were fed pea aphids (*Acyrthosiphon pisum* Harris (Hemiptera: Aphididae) reared on *Vicia faba* L. (Fabales, Fabaceae)) three to four times per week. Eggs and aphids were provided by Koppert Biological Systems, Berkel en Rodenrijs, the Netherlands.

Experiment

The experiments were conducted on twelve young lime trees (*Tilia pallida*) in the orchard of the experimental farm Droevendaal, Wageningen University, Wageningen, the Netherlands. The trees were trimmed and trained in espalier form making the branches easily accessible for the observer (appendix 2a); branches were trained in a North-South direction. Trees were planted in a 3*4 matrix with 3 meters between the trees. Each tree was enclosed by a cage (2.5 x 2 x 3.2m), see photo page 64. The soil was covered with natural vegetation of grasses and weeds. Ladybirds and other insects interfering with aphids (e.g. ants, lacewings, hoverflies, Lepidopteran caterpillars) were removed before and during the experiments. Tape covered with olive oil around stems and supporting poles prevented ants from climbing into the trees.

Trees were infested by adding aphid-infested branches (*Eucallipterus tiliae*). Once established, aphid populations grew rapidly, resulting in aphid populations large enough to support ladybird development. If necessary infested branches were added. Inside two cages data loggers (MicrologPRO, Fourier Systems) recorded the temperature every half hour.

To determine development and survival of *H. axyridis* and *A. bipunctata* larvae were placed on the trees in three treatments: 1) only *H. axyridis* larvae; 2) only *A. bipunctata* larvae; and 3) combined, i.e. equal numbers of larvae of both *H. axyridis* and *A. bipunctata* to test the effect of interspecific competition. Three trees were used per treatment while the three remaining trees served as controls and did not receive larvae. The treatments were randomly assigned to the trees, with the restriction that each treatment contained one lush and one more sparsely leafed tree, as six trees were lusher than the other six. The experiment was repeated five times: starting 22 May (I), 15 June (II), and 7 July 2009 (III), and 8 June (IV) and 1 July 2010 (V). In period I six larvae were placed on each tree. In the following periods six larvae were placed on each of the six more sparsely leafed trees and ten larvae on each of the six lusher trees.

The experiments were started with freshly hatched larvae that had not yet dispersed from their egg batch, except for period V, when the larvae were one day old. Larvae were taken from three (sparsely leafed trees) or five (lush trees) different parental pairs, to avoid family effect. Each larva was placed on a leaf in the vicinity of aphids. Every other day the developmental stage of the larvae was recorded and their position labelled. Skins that were shed after ecdysis were recorded as well, and removed. Developmental stage of larvae and skins was determined based on colour (Hodek 1973; Koch 2003; Stippen.nl 2009) and reared reference individuals.

Larvae and pupae were often difficult to find in this non-destructive experiment and hence not all individuals were always found. Therefore, the number of individuals successfully moulting into the next stage and the day on which that happened was

determined on the basis of both the number of observed individuals and on the number of shed skins of the earlier stages found. To determine the number surviving to a next stage, the number of individuals in later stages was also taken into account. When larvae moulted into pupae the size of the pupa was measured with an electronic digital caliper (mm ± 0.1 mg, ETC Tools) and a clip cage was placed over the pupa (appendix 2b). After emergence adults were weighed on a micro balance (mg ± 0.1 mg, Satorius and Mettler Toledo). Two extremely small *H. axyridis* adults (outliers, period V) were removed from the analysis of pupal size and weight.

Statistical analysis

To estimate developmental time, data were pooled over trees per combination of period and species. The average duration of the larval stages and their standard errors were estimated with the non-parametric method of Pontius et al. (1989) performed by a programme for the analysis of single cohort stage-frequency data written by Manly (1994). For analysis, the development times of the fourth larval instar and the prepupal stage were summed and standard errors were appropriately corrected.

To determine survival, the data were pooled into three groups: L1–L3, the first three instars (L1+L2+L3), i.e. percentage of first instar larvae that successfully moulted into the fourth larval stage; L4PP, the fourth instar (including prepupa, L4+PP), i.e. percentage of fourth instar larvae that successfully moulted into the pupal stage; and P, Pupa, i.e. percentage of pupae that successfully emerged as adult. Overall immature survival was determined as well. For further analyses survival proportions were arcsine-square root transformed. Data on adult weight were square root transformed.

The effect of species, interspecific competition, and, if applicable, year and sex, on development, survival, pupal size, and adult weight was determined using a mixed linear model (procedure MIXED of SAS/STAT® software, version 9.2 (SAS Institute Inc., 2008)). The factors species, competition, and, if applicable, year and sex, were included in the mixed model with fixed effects. Relevant interactions between these factors were added. Random effects for period within year, and if applicable, for tree within year and for period-tree combination within year were included in the mixed model to follow the trial design as closely as possible. In the case of development data, data were pooled over trees; therefore random effects for period-tree pool combination within year were used. Degrees of freedom for F-tests of fixed effects were estimated using the method of Kenward-Roger (Kenward and Roger 1997).

When data were pooled, a weight factor was applied: developmental data were weighted with the $1/(\text{standard error})^2$, where the standard error was estimated simultaneously with the developmental time; survival data were weighted with N, i.e. the number of larvae started with at the beginning of each period. The best models were selected with Akaike's Information Criterion (AIC) (Bolker 2008).

Results

Development

The development time from L1 to adult of *H. axyridis* was significantly longer than that of *A. bipunctata* (table 1, figure 2; P = 0.007, table 2). Analysis of the individual stages shows that this difference is also found for the fourth larval instar (P = 0.002; table 2),

while for the other stages no species effect was observed. No evidence for effects of year, combined-treatment, or of the interactions year*species and combined-treatment*species was found. Inter-period variance was large, except for the first instar. P-values, F-values, and estimates of the variance components are given in table 2. For the *A. bipunctata*-single treatment in period I the prepupa and pupa stages were not found; therefore development time could not be estimated, although at the end of the experiment two freshly emerged adults were found.

Table 1 *Immature development (days (SE)) of* Harmonia axyridis *and* Adalia bipunctata *in the field on lime trees with a single species or both species combined on a tree. L4PP = fourth instar including prepupa; L1toA = L1 until adult emergence.*

Year	**2009**						**2010**			
Period	**I**		**II**		**III**		**IV**		**V**	
Treatment	**Single**	**Combined**	**Single**	**Combined**	**Single**	**Combined**	**Single**	**Combined**	**Single**	**Combined**
					Harmonia axyridis					
larva 1	5.0 (0.4)	4.0 (0.00)	4.1 (0.11)	4.0 (0.0)	5.4 (0.2)	5.7 (0.3)	4.9 (0.2)	4.8 (0.3)	3.0 (0.0)	3.0 (0.0)
larva 2	4.2 (0.5)	4.8 (0.44)	4.0 (0.24)	4.1 (0.4)	2.6 (0.2)	2.7 (0.4)	2.6 (0.3)	3.2 (0.3)	2.0 (0.0)	2.0 (0.0)
larva 3	3.9 (0.5)	4.2 (0.75)	2.7 (0.33)	2.6 (0.5)	2.9 (0.3)	1.6 (0.3)	3.2 (0.3)	2.4 (0.3)	2.2 (0.1)	2.0 (0.0)
larva 4	7.4 (0.5)	9.0 (0.77)	4.4 (0.32)	3.9 (0.3)	7.0 (0.4)	7.3 (0.3)	6.2 (0.3)	6.9 (0.3)	4.2 (0.2)	4.0 (0.2)
prepupa	2.5 (0.4)	2.0 (0.56)	1.0 (0.20)	1.4 (0.2)	1.7 (0.2)	2.0 (0.3)	1.0 (0.2)	1.0 (0.3)	0.6 (0.1)	0.5 (0.2)
L4PP	9.8 (0.8)	11.0 (1.1)	5.4 (0.4)	5.3 (0.5)	8.7 (0.5)	9.2 (0.5)	7.2 (0.4)	7.9 (0.5)	4.7 (0.3)	4.5 (0.3)
larva	22.9 (0.3)	24.0 (0.5)	16.2 (0.2)	16.0 (0.2)	19.6 (0.2)	19.2 (0.2)	17.9 (0.2)	18.3 (0.2)	11.9 (0.1)	11.5 (0.0)
pupa	8.3 (0.5)	7.8 (0.6)	5.0 (0.25)	5.1 (0.3)	6.7 (0.3)	6.8 (0.3)	5.8 (0.2)	5.0 (0.3)	6.1 (0.2)	6.2 (0.2)
L1toA	31.4 (0.3)	31.8 (0.4)	21.2 (0.18)	21.0 (0.2)	26.4 (0.2)	26.0 (0.2)	23.6 (0.2)	23.3 (0.3)	18.0 (0.2)	17.7 (0.2)
					Adalia bipunctata					
larva 1	5.9	4.0 (0.0)	4.0 (0.0)	4.3 (0.3)	5.5 (0.2)	5.7 (0.3)	4.8 (0.3)	5.0 (0.4)	3.1 (0.1)	4.0 (0.4)
larva 2	2.1	4.0 (0.0)	3.0 (0.4)	3.4 (0.5)	3.1 (0.6)	2.6 (0.4)	3.8 (0.4)	2.8 (0.5)	1.7 (0.2)	1.0 (0.4)
larva 3	5.3	4.0 (0.0)	3.2 (0.4)	2.5 (0.6)	2.0 (0.0)	2.2 (0.5)	2.5 (0.6)	2.6 (0.5)	2.4 (0.3)	2.0 (0.0)
larva 4	4.2	6.5 (0.4)	3.0 (0.3)	3.7 (0.3)	6.2 (0.7)	6.3 (0.6)	5.7 (0.6)	6.0 (0.5)	2.7 (0.3)	3.0 (0.3)
prepupa	-	2.3 (0.7)	2.1 (0.4)	1.3 (0.3)	1.3 (0.4)	1.6 (0.46)	1.1 (0.4)	1.1 (0.3)	1.4 (0.3)	1.1 (0.3)
L4PP	-	8.8 (0.9)	5.1 (0.6)	5.0 (0.4)	7.4 (0.9)	7.9 (0.9)	6.8 (0.9)	7.1 (0.7)	4.1 (0.5)	4.1 (0.5)
larva	-	20.8 (0.6)	15.3 (0.3)	15.3 (0.2)	18.0 (0.4)	18.4 (0.5)	18.0 (0.3)	17.5 (0.0)	11.3 (0.2)	11.1 (0.2)
pupa	-	8.3 (0.8)	5.2 (0.4)	5.3 (0.3)	6.0 (0.5)	6.4 (0.6)	5.9 (0.4)	6.3 (0.2)	4.9 (0.3)	5.2 (0.3)
L1toA	-	29.2(0.5)	20.5 (0.3)	20.5 (0.2)	24.9 (0.2)	24.8 (0.4)	23.9 (0.3)	23.8 (0.2)	16.2 (0.2)	16.3 (0.2)

Table 2 *Immature development time was analysed with mixed models; the null hypotheses of no effect were tested for fixed effects. P-values and F-values of fixed effects and estimates of the variance components. Significant P-values are given in bold.*

Development	**L1**		**L2**		**L3**		**L4PP**		**pupa**		**L1 to adult**	
fixed effects	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
year	$F_{1,4.1}$=0.26	0.64	$F_{1,1.9}$=0.87	0.45	$F_{1,2.9}$=0.50	0.53	$F_{1,45.0}$=0.37	0.57	$F_{1,2.9}$=0.70	0.47	$F_{1,3.0}$=1.44	0.32
species	$F_{1,10.0}$=0.80	0.40	$F_{1,10}$=2.34	0.16	$F_{1,10.0}$=0.00	0.995	$F_{1,10.4}$=17.5	**0.002**	$F_{1,9.9}$=0.21	0.66	$F_{1,10}$=11.4	**0.007**
competition	$F_{1,12.6}$=0.08	0.78	$F_{1,10.3}$=0.01	0.91	$F_{1,10.1}$=4.19	0.07	$F_{1,6.4}$=1.13	0.33	$F_{1,9.9}$=0.06	0.81	$F_{1,10}$=0.01	0.93
year*species	$F_{1,9.9}$=1.43	0.26	$F_{1,9.9}$=0.16	0.69	$F_{1,10.0}$=0.07	0.80	$F_{1,9.2}$=0.48	0.51	$F_{1,9.9}$=0.44	0.52	$F_{1,10}$=0.32	0.58
combined*species	$F_{1,9.7}$=1.41	0.26	$F_{1,10.0}$=1.37	0.27	$F_{1,10.1}$=1.43	0.26	$F_{1,10.4}$=0.03	0.86	$F_{1,9.9}$=0.49	0.50	$F_{1,10}$=0.25	0.63
random effects												
period(year)	0		0.47		0.65		2.95		1.42		23.75	
treepool*period(year)	0.013		0.0029		0		0.027		0		0	
residual	0.59		0.84		0.54		0.26		1.14		5.80	

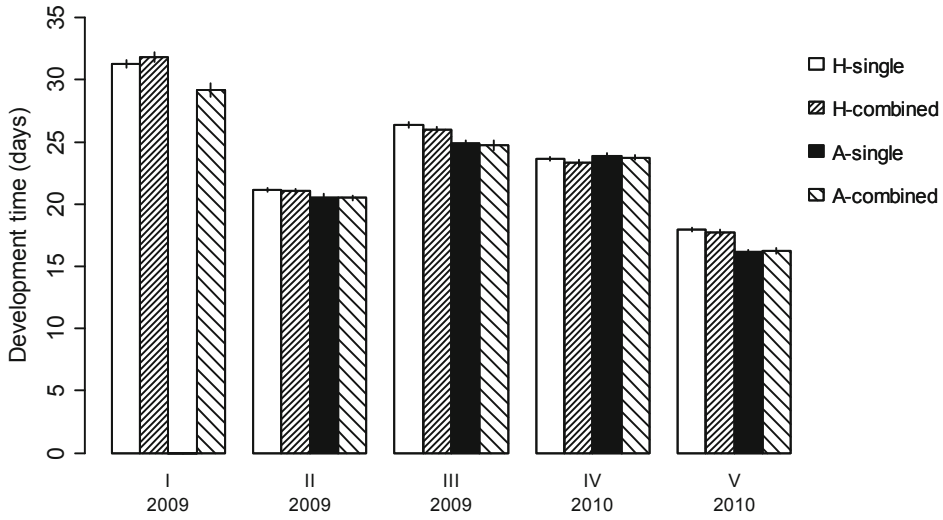


Figure 1 *Development time from L1 to adult emergence (days (SE)) of* Harmonia axyridis *(H) and* Adalia bipunctata *(A) in the field on lime trees in five periods (I–V) with a single species or both species combined on a tree.*

Survival

Under field conditions, but with the exclusion of natural enemies, immature survival (from L1 to adult emergence) of *H. axyridis* ranged from 44.4% to 100% over the different periods, while immature survival of *A. bipunctata* ranged from 11.1% to 76.9% (figure 2, table 3). Averaged over the two years, survival of *H. axyridis* was significantly higher than that of *A. bipunctata* (P = 0.0014, table 4), but the difference in survival rate was not constant over years (interaction year*species, P = 0.0078). In 2010 survival of *H. axyridis* and *A. bipunctata* was similar.

Statistical analysis of stage-specific survival, shows that only survival of L1–L3 differed between species (P < 0.0001) and that only pupal survival had a significant interaction of year*species (P = 0.0007). For none of the stages evidence was found for an effect of combined-treatment, which is similar to what was found for overall immature survival. For all stages as well as for overall immature survival, the variance component for period was consistently estimated to be positive, whereas variance components for tree and period*tree were bound at 0 in some cases (table 4). Variability between periods within years was considerable for all stages; AIC showed that this is an important factor (results not shown). P-values, F-values, and estimates of the variance components are given in table 4.

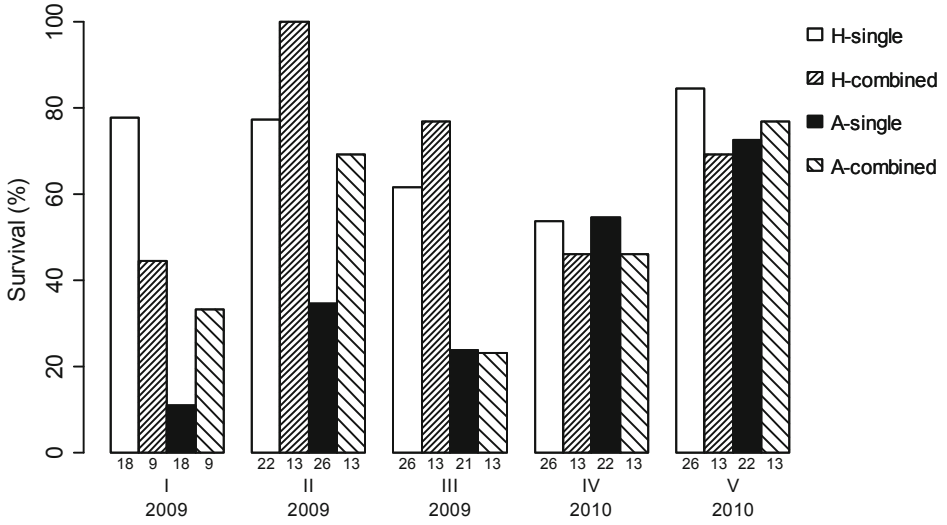


Figure 2 *Immature survival from L1 to adult emergence of* Harmonia axyridis *(H) and* Adalia bipunctata *(A) in the field on lime trees with a single species or both species combined on a tree in five periods (I–V). Number of individuals for each treatment is given below each bar.*

Table 3 *Immature survival of* Harmonia axyridis *and* Adalia bipunctata *in the field on lime trees with a single species or both species combined on a tree. N = Number of individuals per treatment. L1–L3 = first three instars (L1+L2+L3); L4PP = fourth instar including prepupa; L1toA = L1 until adult emergence.*

Year	**2009**										**2010**									
Period	**I**				**II**				**III**				**IV**				**V**			
Treatment	**Single**		**Combined**		**Single**		**Combined**		**Single**		**Combined**		**Single**		**Combined**		**Single**		**Combined**	
Harmonia	**mean**	**N**	**mean**	**N**	**mean**	**N**	**mean**	**N**	**mean**	**N**	**mean**	**N**	**mean**	**N**	**mean**	**N**	**mean**	**N**	**mean**	**N**
L1–L3	77.8	18	44.4	9	81.8	22	100.0	13	69.2	26	100.0	13	76.9	26	76.9	13	88.5	26	92.3	13
L4PP	100.0	14	100.0	4	94.4	18	100.0	13	88.9	18	76.9	13	100.0	20	90.0	10	95.7	23	75.0	12
pupa	100.0	14	100.0	4	100.0	17	100.0	13	100.0	16	100.0	10	70.0	20	66.7	9	100.0	22	100.0	9
L1toA	77.8	18	44.4	9	77.3	22	100.0	13	61.5	26	76.9	13	53.8	26	46.2	13	84.6	26	69.2	13
Adalia	**mean**	**N**	**mean**	**N**	**mean**	**N**	**mean**	**N**	**mean**	**N**	**mean**	**N**	**mean**	**N**	**mean**	**N**	**mean**	**N**	**mean**	**N**
L1–L3	16.7	18	33.3	9	50.0	26	84.6	13	31.8	22	46.2	13	54.5	22	53.8	13	77.3	22	76.9	13
L4PP	66.7	3	100.0	3	100.0	13	90.9	11	85.7	7	50.0	6	100.0	12	100.0	7	94.1	17	100.0	10
pupa	100.0	2	100.0	3	69.2	13	90.0	10	83.3	6	100.0	3	100.0	12	85.7	7	100.0	16	100.0	10
L1toA	11.1	18	33.3	9	34.6	26	69.2	13	23.8	21	23.1	13	54.5	22	46.2	13	72.7	22	76.9	13

Table 4 *Immature survival was analysed with mixed models; the null hypotheses of no effect were tested for fixed effects. P-values and F-values of fixed effects and estimates of the variance components. Significant P-values are given in bold. L1–L3 = first three instars (L1+L2+L3); L4PP = fourth instar including prepupa; L1toA = L1 until adult emergence.*

Survival	**L1–L3**		**L4PP**		**pupa**		**L1toA**	
Fixed effects	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
year	$F_{1,2.9}$=1.01	0.39	$F_{1,4.6}$=0.62	0.47	$F_{1,3.3}$=0.01	0.92	$F_{1,3.6}$=0.99	0.38
species	$F_{1,32.1}$=25.1	<0.0001	$F_{1,40.5}$=0.01	0.93	$F_{1,46.7}$=0.25	0.62	$F_{1,42}$=13.9	0.0006
competition	$F_{1,30.2}$=3.37	0.08	$F_{1,47.6}$=1.83	0.18	$F_{1,46.4}$=0.49	0.49	$F_{1,50.5}$=0.68	0.41
year*species	$F_{1,43.9}$=2.63	0.11	$F_{1,42.2}$=3.5	0.07	$F_{1,46.8}$=13.02	0.0007	$F_{1,46.3}$=9.51	0.003
combined*species	$F_{1,31.5}$=0.00	0.99	$F_{1,37.6}$=0.32	0.57	$F_{1,46.6}$=0.51	0.48	$F_{1,42.3}$=0.64	0.43
Random effects								
tree(year)	0		0.025		0		0.023	
period(year)	0.033		0.014		0.017		0.036	
tree*period(year)	0.020		0		0		0	
residual	0.38		0.23		0.22		0.55	

Size and weight

Harmonia axyridis pupae were larger and adults were heavier than those of *A. bipunctata* (table 5; P < 0.0001, table 6). In both species females were larger and heavier than males (P < 0.0001, table 6). Regarding pupal length the sex difference was smaller in *A. bipunctata* than in *H. axyridis* (interaction species*sex, P = 0.032). No overall differences between the two years were found, but with regard to pupal width the species difference was larger in 2009 than in 2010 (interaction year*species, P = 0.02). No effect

Table 5 *Length and width of pupae (mm and SD) and adult weight (mg and SD) of* Harmonia axyridis *and* Adalia bipunctata *in the field on lime trees with a single species or both species combined on a tree. F = female, M = male.*

	Year		**2009**										**2010**							
	Period		**I**				**II**				**III**				**IV**				**V**	
	Treatment		**Single**		**Combined**		**Single**		**Combined**		**Single**		**Combined**		**Single**		**Combined**		**Single**		**Combined**	
	Harmonia	**sex**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**
Pupa	**Width**	**mean**	4.44	4.45	4.34	4.24	4.39	4.23	4.53	4.27	4.02	3.87	4.04	3.87	4.59	4.29	4.57	4.21	4.38	4.23	4.55	4.15
	(mm)	**SD**	0.14	0.03	0.17	0.13	0.17	0.13	0.15	0.11	0.20	0.12	0.08	0.08	0.17	0.05	0.13	0.09	0.20	0.11	0.11	0.17
	Length	**mean**	6.06	5.74	5.80	5.56	6.02	5.84	6.17	5.78	5.58	5.33	5.59	5.34	6.25	5.91	6.20	5.90	5.95	5.77	6.08	5.66
	(mm)	**SD**	0.14	0.13	0.00	0.06	0.26	0.21	0.22	0.11	0.23	0.22	0.12	0.09	0.20	0.13	0.23	0.18	0.26	0.19	0.04	0.15
		N	4	2	2	2	8	9	6	7	9	5	5	4	6	8	4	2	11	9	2	7
Adult	**Weight**	**mean**	35.7	32.5	32.2	28.7	35.6	32.8	32.4	37.6	28.2	24.1	27.5	26.8	37.1	31.3	37.7	29.3	35.8	31.7	37.9	31.1
	(mg)	**SD**	1.6	3.0	3.0	1.7	4.8	3.6	2.1	4.0	3.1	2.7	3.1	2.4	2.2	1.4	1.7	5.0	4.9	2.3	0.8	2.3
		N	6	4	2	2	8	9	7	6	9	5	5	4	5	8	4	2	11	9	2	7
	Adalia	**sex**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**
Pupa	**Width**	**mean**	-	-	3.02	-	3.21	2.92	3.20	-	2.90	2.76	2.70	-	3.27	3.15	3.40	3.15	3.26	3.09	3.33	3.14
	(mm)	**SD**	-	-	-	-	0.29	0.15	0.15	-	0.09	-	0.14	-	0.13	0.03	0.12	0.09	0.12	0.11	0.08	0.13
	Length	**mean**	-	-	4.26	-	4.50	4.26	4.66	-	4.29	4.33	4.12	-	4.60	4.58	4.53	4.33	4.60	4.44	4.70	4.43
	(mm)	**SD**	-	-	-	-	0.33	0.14	0.19	-	0.10	-	0.28	-	0.17	0.08	0.03	0.01	0.12	0.13	0.07	0.22
		N	-	-	1	-	2	4	8	-	4	1	3	-	4	2	2	2	6	8	3	5
Adult	**Weight**	**mean**	10.8	8.6	8.2	10.4	14.5	10.8	13.7	-	11.2	9.3	-	9.1	13.7	11.1	14.0	11.9	14.3	12.4	14.3	12.2
	(mg)	**SD**	-	-	-	0.4	2.4	1.4	1.6	-	0.6	-	-	0.8	1.7	2.2	1.3	0.7	2.0	0.6	1.9	1.2
		N	1	1	1	2	3	5	9		4	1	-	3	5	6	3	3	7	9	5	5

of interspecific competition was found. For all three variables inter-period variance was large. P-values, F-values, and estimates of the variance components are given in table 6.

Table 6 *Size and weight were analysed with mixed models; the null hypotheses of no effect were tested for fixed effects. P-values and F-values of fixed effects and estimates of the variance components. Significant P-values are given in bold.*

Size and weight	**Pupal length**		**Pupal width**		**Adult weight**	
Fixed effects	**F**	**P**	**F**	**P**	**F**	**P**
year	$F_{1,3.3}$=2.00	0.24	$F_{1,3.0}$=1.65	0.29	$F_{1,3.1}$=1.81	0.27
species	$F_{1,151}$=1921.3	**<0.0001**	$F_{1,80.2}$=2190.3	**<0.0001**	$F_{1,65.4}$=2337.7	**<0.0001**
sex	$F_{1,155}$=50.8	**<0.0001**	$F_{1,154}$=61.3	**<0.0001**	$F_{1,153}$=66.7	**<0.0001**
competition	$F_{1,99.8}$=0.04	0.85	$F_{1,23.7}$=0.23	0.64	$F_{1,14.8}$=0.15	0.71
year*species	$F_{1,148}$=0.00	0.99	$F_{1,76.7}$=5.45	**0.02**	$F_{1,52.4}$=2.11	0.15
species*sex	$F_{1,155}$=4.66	**0.032**	$F_{1,155}$=1.08	0.30	$F_{1,155}$=0.07	0.79
competition*species	$F_{1,155}$=0.02	0.89	$F_{1,57.7}$=0.05	0.82	$F_{1,43.4}$=0.65	0.42
Random effects						
tree(year)	0.0063		0.00094		0.0034	
period(year)	0.30		0.032		0.068	
tree*period(year)	0		0.0014		0.0024	
tree(year)	0.027		0.016		0.053	

Temperature

The average temperature in the cages during the experiments ranged from 18.0 (I) to 23.8°C (V) (figure 7), which is on average 2.6°C higher than the temperature outside the cages. Especially peak temperatures were high in the cages (maximum ranges from 36.0 to 43.2°C), which is on average 8.4°C higher than outside temperatures.

Table 7 *Average, minimum and maximum temperature measured during the experiments in the cages and in a nearby weather station (KNMI-Deelen 2013), and minimum and maximum day length (AA-USNO 2013)*

Year	**Period**	**Date**		**Average**	**Range**	**Min–max day length (hour:minutes)**
2009	I	22 May–27 June	cage	18.0	(3.2–36.0)	16:01–16:44
			outside	14.9	(2.0–26.9)	
	II	15 June–11 July	cage	20.7	(5.1–40.0)	16:23–16:44
			outside	17.5	(3.6–30.9)	
	III	7 July–6 August	cage	19.8	(8.1–38.6)	15:11–16:30
			outside	17.4	(6.9–28.9)	
2010	IV	8 June–3 July	cage	19.9	(4.3–40.8)	16:36–16:44
			outside	17.6	(3.9–34.9)	
	V	1 July–21 July	cage	23.8	(7.0–43.2)	16:01–16:38
			outside	21.7	(5.4–35.0)	

Discussion

Development

This study is the first to determine development and survival characteristics of ladybird species under field conditions in northwestern Europe. Two field studies are known from other areas, i.e. France and Japan. In southern France development from L1 to adult emergence of *H. axyridis* was determined in outdoor rearing cages with artificial food (Ongagna et al. 1993). In Japan *H. axyridis* and *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) were observed on trees in a botanical garden from mid-May to mid-July (Kindlmann et al. 2000) and larval development was estimated using the method of Manly (1997).

The development times of *H. axyridis* found in our study fall within the range of available data on development times determined under laboratory conditions (chapter 8) (table 8). The development times found at comparable temperatures in the two other field studies were either much longer (France, Ongagna et al. 1993) or considerably shorter (Japan, Kindlmann et al. 2000) (table 9), while both were performed under shorter day length than our study. We do not have an explanation for these differences.

Table 8 *Development time from L1 to adult emergence of* Harmonia axyridis *found in current study and estimatedmodel estimates of development time based on laboratory studies of* H. axyridis *reared under long-day conditions at the same average temperature. The model did not discriminate between origin, food, and ladybird strain (chapter 8).*

		Development (days)		
		Current study		Model estimate
Period	Temperature	Single	Combined	
I	18.0	31.3	31.8	29.5
II	20.7	21.2	21.0	22.1
III	19.8	26.4	24.0	24.1
IV	19.9	23.6	23.3	23.9
V	23.8	18.0	17.7	17.2

In this study, development times were determined in cages, where on average temperature was 2.6°C higher than outside, so development outside the cages in spring in the Netherlands will be slower than in the cages. Based on a laboratory study of Krengel et al. (2012) where a 3°C higher average temperature in June and July resulted in a 6 to 8 days faster development, we assume that development time under spring conditions would be about a week longer than that determined in this study.

The observed development times from L1 to adult emergence for *A. bipunctata* are quite long compared to previous studies (table 6 and figure 3), but all literature data are determined in the laboratory with reared populations of *A. bipunctata*. In general captivity rearing selects for shorter developmental times than found in the field (Nunney 2003; Tayeh et al. 2012), so this might explain the difference in the development time we found. When comparing the data on reared populations for the stages separately, pupal development is similar while our larval development time is longer than found in the literature. The few development times determined with field

Table 9 *Development of* Harmonia axyridis *under field conditions in France (a. L1 to adult emergence, Ongagna et al. 1993) and Japan (b. larva, Kindlmann et al. 2000). Sources for weather data: France, day length (AA-USNO 2013); Japan, temperature (JMA 2013) and day length (JCG/HOD 2013).*

Period	Average temperature (°C)°	Development (days)	Min–max day length (hour:minutes)
a. France		**L1 to adult**	
May 1988	16.6	37	14:10–15:12
June 1988	19.1	31	15:13–15:26
July 1988	22.2	28	15:22–14:35
August 1988	23.3	18	14:33–13:14
September 1988	19.2	23.7	13:11–11:45
b. Japan		**Larva**	
May–July 1993	18.2	17.4	14:17–14:52
May–July 1994	19.9	16.5	14:17–14:52
May–July 1995	18.9	16.2	14:17–14:52
May–July 1996	18.4	16.7	14:17–14:52

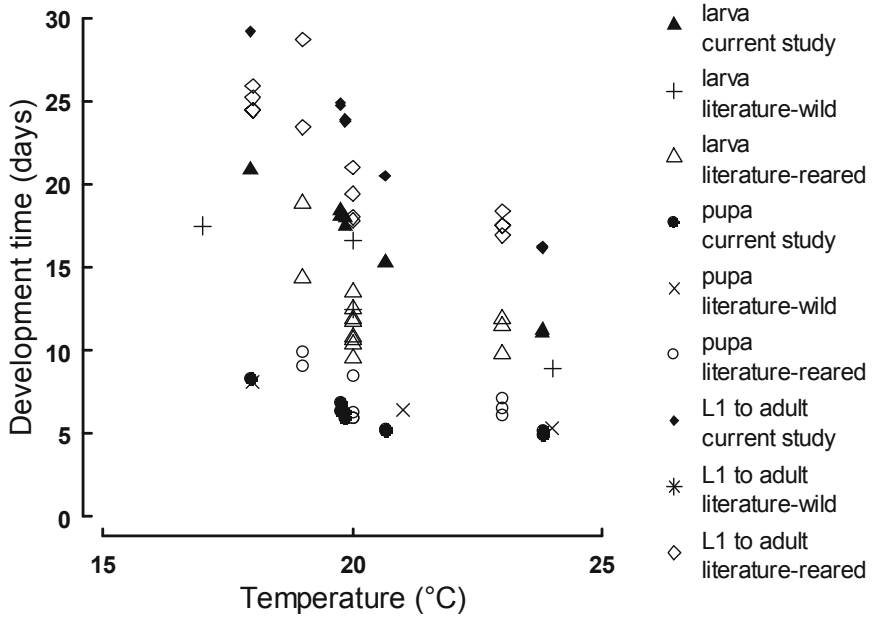


Figure 3 *Development time of* Adalia bipunctata *from L1 to adult emergence and for the larval and pupal stage separately. Current study and data from literature based on reared or wild populations. For exact values and references see appendix.*

collected populations for both larval and pupal stages are similar to values found in this study.

In general *H. axyridis* develops slower than *A. bipunctata*. This is in accordance with the lower developmental threshold for *A. bipunctata* (8.5–10.1°C) compared with that of *H. axyridis* (10.5–11.2°C), while their thermal constants are comparable (*A. bipunctata* 244.8–67.9 versus *H. axyridis* 231.3–258.3 and 286.5 from model estimation (chapter 8)). Analysis of the development time of the individual immature stages shows that only the difference in development between the species of L4PP stage determines observed difference in overall development time.

Survival

Survival of *A. bipunctata* was significantly higher in 2010 than in 2009. This difference may be explained by two factors: 1) In 2010 offspring of wild collected individuals was used instead of offspring from a laboratory stock culture and 2) for period V in 2010 one-day-old first instar larvae were used, that had had the opportunity to feed on sibling eggs before release and were therefore probably less fragile than freshly hatched larvae. Overall immature survival of *H. axyridis* was higher than that of *A. bipunctata.* For individual stages, only the L1–L3 stages show differences in survival between species.

When prey is limited, mortality of the larval stages is the key factor determining population dynamics of *H. axyridis* in the field (Osawa 1993), in particular the fourth instar (Kindlmann et al. 2000). Osawa (1993) attributed mortality of younger stage larvae to their low prey-searching and capturing ability (Kawai 1978) even when aphid density was high, and attributed fourth-instar mortality to absolute food shortage (Osawa 1992b). We did not observe high mortality of the fourth larval stage, probably because we used high prey densities. Factors causing considerable larval mortality, other than intraguild predation, are unknown. In this study natural enemies were excluded, but mortality caused by natural enemies is usually low (Osawa 1993; Yasuda and Shinya 1997), although some predation of eggs and younger larval stages by ants has been reported (Burgio et al. 2008).

In this experiment larvae were placed on the leaf individually while both *H. axyridis* and *A. bipunctata* lay their eggs in clusters. Hemptinne et al (2000) showed that first-instar larvae are attracted to aphids eaten by conspecific larvae and argued that this increased their chance of finding prey and surviving because siblings are near each other since eggs are laid in clusters. This could indicate that our study underestimates first-instar survival under conditions of high prey-density.

In literature reported survival rates at temperatures above 30°C are variable: no survival at 30°C, 32°C (Michaud 2002b), and 35°C (Wang et al. 2009); reduced survival at 30°C (22–56%, Lombaert et al. 2008), 33°C (50%, Knapp and Nedved 2013), and 34°C (25%, LaMana and Miller 1998); and full development at 30°C (larvae and pupae, Kawauchi 1979) and 38°C (pupae, Knapp and Nedved 2013). In the current study in all periods high temperatures around or above 35°C were reached at some point during the experiments and many larvae and pupae did survive these temperatures. In contrast to the laboratory conditions, temperatures in our field study fluctuated and larvae are apparently able to survive periods with high temperatures. Two studies

confirm that most instars tolerate high temperatures for a short time: 48 hours at 33°C (Knapp and Nedved 2013) and two hours at 35°C and 40°C (Acar et al. 2004).

In the current study pupal survival is almost always 100%, except during period II (2009) and IV (2010) when five and ten individuals died. These exceptions of pupal mortality account for the significant interaction year*species we found in the pupal stage. Knapp and Nedved (2013) found that the pupa stage was most resistant to a period of raised temperature of 33°C for 48 hours. This suggests that the high mortality is not the result of the relatively long period of high temperatures of around 35°C observed during pupal development in period II and IV.

Size and weight

Adult weight of *A. bipunctata* is in line with previous studies (table 10), while the weight of *H. axyridis* is within the upper range of literature data on aphid-fed populations. Food quality and quantity can affect adult size considerably (Hodek and Honek 1996), but food quality and quantity were not limited in this study, hence we have no explanation for the high weight observed for *H. axyridis*.

Table 10 *Literature data on adult weight (mg (range)) at emergence of European, aphid-fed populations of* Harmonia axyridis *and* Adalia bipunctata*.*

Harmonia axyridis				
Population	**Temperature**	**Female weight (mg)**	**Male weight (mg)**	**Reference**
wild	23°C	39.2 (38.0–40.6)	32.3 (31.4–33.2)	Berkvens et al. 2008b
	25°C	24.3 (18.5–33)	21.3 (15.1–29.5)	Ungerova et al. 2010
		23.7 (20.7–25.4)	18.6 (18.2–19.2)	Nedved and Kalushkov 2012
reared	15°C	23.3	22.6	Schanderl et al. 1985
	17.8 °C	31.3	25.7	Krengel et al. 2012[a]
	20°C	32.0	26.7	Schanderl et al. 1985
		33.6 (30.6–36.5)	29.4 (27–31.8)	Soares et al. 2001
	20.8°C	28.8	23.3	Krengel et al. 2012[a]
	23°C	31.3 (30.6–32.0)	27.9 (27.2–28.6)	Berkvens et al. 2008b
		32	30	Kögel et al. 2012a
	25°C	29.6	26.7	Schanderl et al. 1988
		30.5	26.9	Schanderl et al. 1985
	30°C	32.2	28.7	Schanderl et al. 1985
Adalia bipunctata				
wild	20°C	12.1	10.5	Yasuda and Dixon 2002
reared	18°C	11.5 (10.1–12.9)	9.5 (8.9–10.0)	Ferrer et al. 2008
	19°C	13.2	10.1 (10.0–10.0)	Jalali et al. 2009
	20°C	10.7		Blackman 1967
	22°C	14.1 (13.7–14.6)		Kajita et al. 2006b
	23°C	13.28 (13.2–13.4)	10.3 (10.2–10.4)	Jalali et al. 2009
	27°C	11.64 (11.5–11.8)	8.8 (8.7–8.9)	Jalali et al. 2009

[a] *based on figure 2, data extracted with Engauge digitalizer*

Effect of interspecific competition

Under conditions of low prey availability, intraguild predation is an important force structuring the guild of ladybirds (Yasuda and Shinya 1997). In our study no evidence was found for an effect of intraspecific competition. However, for L3 development and L1–L3 survival differences between treatments with conspecifics only and the combined-treatment with both species are nearly significant. Hence, species in this study did not interfere to such an extent that differences were detectable. Although intraguild predation under field conditions has been found (chapter 6, Gagnon et al. 2011; Hautier et al. 2011; Thomas et al. 2013), it seems to be negligible at high prey-densities.

A major part of the observed variation, especially for development from L1 to adult can be explained by differences between periods, which, in turn can be attributed to many factors like variation in temperature, humidity, and the condition of trees and prey.

In conclusion, we were able to provide estimates of development time and survival in the field, despite incomplete life tables. We also show that with ample provision of prey 1) immature development in the field is in line with laboratory data; 2) survival is generally high; and 3) *A. bipunctata* and *H. axyridis* do not negatively interfere; and 4) intraguild predation does not seem to play a role.

Harmonia axyridis has a higher survival and therefore a faster population growth than *A. bipunctata*, which may contribute to its invasion success.

Acknowledgements

Thanks to the following people for various forms of assistance: SW Benedictus-van den Berg, CAJM van Boxel, JMG Haarhuis, L Hemerik, G Mekking, K Oku, and MJ Raak, H van Roekel, and A Siepel.

Appendix 1 chapter 5

Observed data and literature data on developmental time of Adalia bipunctata *from L1 to Adult (larva plus pupa) and for the stages larva and pupa separately. Literature data are determined in the laboratory with reared populations, unless otherwise indicated. References are indicated by a number between brackets and given below.*

L1 to adult

Period		I	II	III	IV		V
Average temperature		17.95°C	20.65°C	19.75°C	19.85°C		23.8°C
Observed		29.17	20.50	24.90	23.91		16.15
			20.50	24.75	23.75		16.25
Literature	18°C	24.43 [1]	20°C	17.8 [2]		23°C	16.9 [3]
		24.45 [1]		18 [2]			17.5 [4]
		25.2 [1]		19.4 [2]			18.37 [5]
		25.9 [1]		21 [6]			17.52 [5]
	19°C	23.41 [5]					
		28.7 [5]					

Larva

Period		I	II	III	IV		V
Average temperature		17.95°C	20.65°C	19.75°C	19.85°C		23.8°C
Observed		20.83	15.28	18.03	18		11.25
			15.25	18.38	17.5		11.07
Literature	17°C	17.48 [7][a]	20°C	9.5 [8]		23°C	9.8 [3]
	19°C	14.38 [5]		10.4 [8]			11.86 [5]
		18.8 [5]		10.6 [8]			11.45 [5]
				10.8 [8]		24°C	8.9 [9][a]
				11.7 [2]			
				11.9 [2]			
				12.44 [7][a]			
				12.5 [6]			
				13.5 [2]			
				16.6 [10][a]			

Pupa

Period		I	II	III	IV		V
Average temperature		17.95°C	20.65°C	19.75°C	19.85°C		23.8°C
Observed		8.33	5.22	6.88	5.91		4.9
			5.25	6.38	6.25		5.18
Literature	18°C	8.1 [11][a]	20°C	5.9 [2]		23°C	6.51 [5]
	19°C	9.03 [5]		5.9 [2]			6.07 [5]
		9.9 [5]		6.3 [2]			7.1 [3]
				8.5 [6]		24°C	5.3 [11][a]
			21°C	6.4 [11][a]			

[a] *based on a wild population*

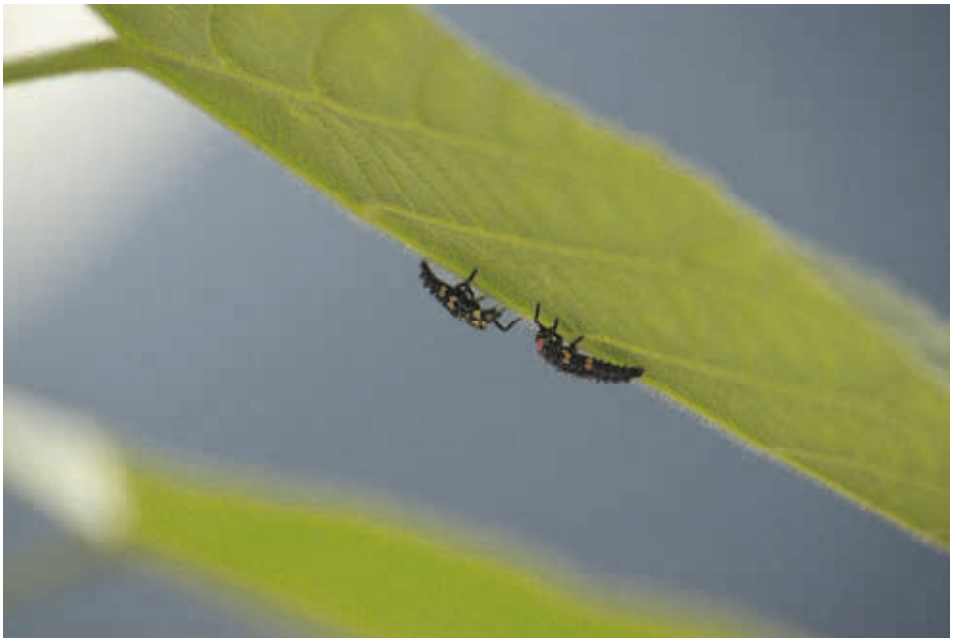
References: [1] Ferrer et al. 2008, [2] Francis et al. 2001, [3] Bonte et al. 2010, [4] De Clercq et al. 2005, [5] Jalali et al. 2009, [6] Schuder et al. 2004, [7] Mills 1981b, [8] Blackman 1967, [9] Kalushkov 1994, [10] Hämäläinen et al. 1975, [11] Honek and Kocourek 1988.

Appendix 2 chapter 5

Espalier formed lime tree in cage (a) and clip cage with pupa (b)

a

b



6

Intraguild predation behaviour of ladybirds in semi-field experiments explains invasion success of *Harmonia axyridis*

PLoS ONE (2012) 7(7): e40681

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Abstract

Harmonia axyridis has been introduced as a biological control agent in Europe and the USA. Since its introduction, it has established and spread, and it is now regarded as an invasive alien species. It has been suggested that intraguild predation is especially important for the invasion success of *H. axyridis*. The aim of this study was to compare the intraguild predation behaviour of three ladybird species (*Coccinella septempunctata, Adalia bipunctata,* and *H. axyridis*). Predation behaviour was investigated in semi-field experiments on small lime trees (*Tilia platyphyllos*). Two fourth instar larvae placed on a tree rarely made contact during 3-hour observations. When placed together on a single leaf in 23–43% of the observations at least one contact was made. Of those contacts 0–27% resulted in an attack. *Harmonia axyridis* attacked mostly heterospecifics, while *A. bipunctata* and *C. septempunctata* attacked heterospecifics as often as conspecifics. In comparison with *A. bipunctata* and *C. septempunctata*, *H. axyridis* was the most successful intraguild predator as it won 86% and 44% of heterospecific battles against *A. bipunctata* and *C. septempunctata* respectively, whilst *A. bipunctata* won none of the heterospecific battles and *C. septempunctata* won only the heterospecific battles against *A. bipunctata. Coccinella septempunctata* dropped from a leaf earlier and more often than the other two species but was in some cases able to return to the tree, especially under cloudy conditions. The frequency with which a species dropped did not depend on the species the larva was paired with. The results of these semi-field experiments confirm that *H. axyridis* is a strong intraguild predator as a consequence of its aggressiveness and good defence against predation from heterospecific species. The fact that *H. axyridis* is such a strong intraguild predator helps to explain its successful establishment as invasive alien species in Europe and the USA.

Introduction

Since its introduction as a biological control agent, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) has established and spread. It is now regarded as an invasive alien species in both Europe and the USA. The ladybird is no longer commercially available in most of Europe (Van Lenteren 2012) as it has a negative impact on non-target insect species, fruit production, and human health (Brown et al. 2008a; Koch and Galvan 2008; Van Lenteren et al. 2008). The invasiveness of *H. axyridis* has also raised concerns about the fate of populations of native coccinellids (Michaud 2002b; Alyokhin and Sewell 2004; Roy et al. 2012) and the impact of this species on the intricate multitrophic aphidophagous food web (Alhmedi et al. 2010).

Harmonia axyridis is cannibalistic and successfully preys upon larvae and eggs of other aphid predators (intraguild predation). It has been suggested that intraguild predation (IGP) is one of the reasons for the success of *H. axyridis* as an invasive species (Pell et al. 2008; Ware and Majerus 2008; Alhmedi et al. 2010). IGP is defined as the killing and eating of species that use similar, often limited, resources and is a well-known phenomenon across a wide range of taxa, such as fish, invertebrates, and mammals (e.g. Polis and Holt 1992). Aphidophagous guilds are systems in which IGP is one of the main forces influencing population structure and dynamics (Lucas 2005; Alhmedi et al. 2010). IGP and cannibalism are suspected to have developed as a result of scarcity or absence of the main prey (Agarwala and Dixon 1992; Sato et al. 2003; 2008). In general, the presence of extraguild prey can reduce the occurrence and intensity of IGP (e.g. Yasuda et al. 2004). Oviposition and larval development of *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) and *Adalia bipunctata* L. (Coleoptera: Coccinellidae) are synchronised with the aphid population peak in northwestern Europe. *Harmonia axyridis*, however, arrives later and has to complete its development when aphid densities are low (Takahashi 1989; Yasuda and Shinya 1997; Jansen and Hautier 2008).

This late arrival of *H. axyridis* is thought to have resulted in a higher dependence on cannibalism and IGP, which probably explains its aggressive nature and successful defence strategies (Takahashi 1989; Yasuda and Shinya 1997; Jansen and Hautier 2008). Indeed, there is strong evidence from laboratory experiments that, within the aphidophagous guild, *H. axyridis* is a strong, if not the strongest intraguild predator (e.g. Lucas 2005; Pell et al. 2008; Ware and Majerus 2008; Alhmedi et al. 2010). Its higher mobility, increased levels of aggressiveness (Yasuda et al. 2001; 2004; Michaud 2002b), and larger size (Felix and Soares 2004; Ware and Majerus 2008) seem to be important factors in the success of *H. axyridis* as an intraguild predator.

Coccinellid larvae can defend themselves against IGP by using a range of behavioural, physiological and morphological strategies, which include; running away or dropping (Sato et al. 2005), release of toxic alkaloids (King and Meinwald 1996), and the presence of features such as dorsal spines (Pell et al. 2008; Ware and Majerus 2008; Hautier et al. 2010). Most evidence for IGP behaviour is based on laboratory experiments (Lucas 2005; Snyder 2009; Weber and Lundgren 2009), but the results are difficult to extrapolate to field conditions due to the increased complexity and variation within and between wild habitats. Structured habitats provide refuge to intraguild prey (Denno and Finke 2006), so intraguild prey suffers less from predation

(Janssen et al. 2007) and the availability of different host plants may also influence IGP pressure (Kajita et al. 2006a). Further, alternative food sources, daily and yearly differences in activity cycles, and the possibilities to avoid confrontation and to escape will reduce IGP events (Weber and Lundgren 2009; Lucas and Rosenheim 2011). As the understanding of all IGP-relations in aphidophagous guilds is hampered by the bias of laboratory experiments, there is a great need for experimental studies under field conditions or semi-field conditions which more closely approximate field conditions than a traditional laboratory setup.

The aim of this study was to compare the IGP-behaviour of three ladybird species under semi-field conditions. Experiments were performed with two native European species (*C. septempunctata* and *A. bipunctata*) and the invasive alien species *H. axyridis*. The three species we studied use different defence mechanisms against IGP. *Coccinella septempunctata* is defended by size (against *A. bipunctata* but not against *H. axyridis* (Ware and Majerus 2008)) by dropping behaviour (Sato et al. 2005) and, to a certain extent, by defensive chemicals (Agarwala and Dixon 1992; Sato and Dixon 2004). *Adalia bipunctata* uses chemical defences, which protect it against *C. septempunctata* (e.g. Agarwala and Dixon 1992; Sato and Dixon 2004), but not completely against *H. axyridis* (Sato and Dixon 2004; Sato et al. 2005). *Harmonia axyridis* defends itself by size (Ware and Majerus 2008), chemical deterrence (reviewed by Sloggett et al. 2011), and morphological structure (spines) (Hautier et al. 2010). Small lime trees (*Tilia platyphyllos* Scop. (Malvales: Malvaceae)) were used as natural host plants for the ladybirds. The following research questions were addressed: 1) how often do two larvae of different or the same species come into contact?; 2) what happens when they make contact?; 3) which species generally wins the interaction?; and 4) can the species be ranked on the basis of the outcome of the interaction? These questions were successfully investigated in two different experimental set-ups: on individual lime tree leaves, where escape responses may affect the interactions, and on whole lime trees, where escape responses along with encounter rates may affect the interactions.

Materials and Methods

Insects

Harmonia axyridis adults were collected from hibernation sites in Kootwijk on 13 November 2009 (location N 51 59 32, E 5 39 43) and Houten on 27 November 2009 (location N 52 1 39, E 5 9 38), the Netherlands. *Adalia bipunctata* adults were also collected at those sites and at various other locations. All *A. bipunctata* individuals were found within aggregations of *H. axyridis*. All collected beetles were kept in a climate cabinet at 5°C ±1, 0:24 L:D to continue overwintering and transferred to a climate chamber at 24°C ±1, 16:8 L:D, 55% ± 5 RH in May 2010. In May and early June 2010, adults of *C. septempunctata* and *A. bipunctata* were collected in Wageningen, the Netherlands (location N 52 10 39, E 5 45 39) and transferred to the same climate chamber at 24°C.

Before the start of the experiments, the beetles were sexed and paired. Eighteen pairs of *H. axyridis*, twenty pairs of *C. septempunctata*, and ten pairs of *A. bipunctata* were formed. Two *C. septempunctata* females and eight *A. bipunctata* females were

laying fertile eggs and were also used. When one adult of a pair died, the surviving adult was paired with a new individual. Each pair or individual female was kept in a Petri dish (Ø 9 cm) lined with filter paper and a folded strip of filter paper as substrate for oviposition. All beetles were given honey water and pollen *ad libitum*. In addition, *C. septempunctata* was daily fed pea aphids (*Acyrthosiphon pisum* Harris (Hemiptera: Aphididae) reared on *Vicia faba* L. (Fabales, Fabaceae)) *ad libitum*, and *H. axyridis* and *A. bipunctata* were fed dead, irradiated eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) *ad libitum* and pea aphids three times a week. Eggs and aphids were provided by Koppert Biological Systems, Berkel en Rodenrijs, the Netherlands.

Egg batches were collected daily. Three to six first instar larvae from each batch were placed individually in Petri dishes and fed with *E. kuehniella* (*H. axyridis* and *A. bipunctata*) or pea aphids (*C. septempunctata*) *ad libitum*. *Coccinella septempunctata* larvae were also given water by means of moistened cotton wool. Third instar *H. axyridis* and *A. bipunctata* larvae were fed pea aphids once. Within 24 hours after moult into the fourth instar, all larvae were starved for 24 hours, with access to water by means of moistened cotton wool.

Starved fourth instar individuals were used in the experiments in six combinations: 1) *H. axyridis* & *H. axyridis*; 2) *C. septempunctata* & *C. septempunctata*; 3) *A. bipunctata* & *A. bipunctata*; 4) *H. axyridis* & *C. septempunctata*; 5) *H. axyridis* & *A. bipunctata*; and, 6. *C. septempunctata* & *A. bipunctata*. For each observation larvae from different parents were combined in order to maximise variation. The larvae were marked with Uni Posca pigment markers (a water-based-acrylic paint) to allow for individual recognition. A pilot test showed that this way of marking allows larvae to move and to develop normally into the next instar. Experiments were conducted in a large cage (4 m x 12 m x 3 m) to keep the trees free from aphid infestation. To avoid disturbance by rain, the roof of the cage was covered with a plastic sheet. Temperature was recorded using a Hobo ProV2 temperature logger (MicroDaq.com, Ltd., Contoocook NH, USA). All experiments were conducted in the period from 4 June to 9 July 2010.

Leaf experiment

The behaviour of two larvae encountering each other on a leaf was observed. Compared to studies performed in Petri dishes, the size of the leaf formed a comparable area for interactions to take place but the larvae had the opportunity to escape, which may be an important outcome of interactions in field conditions. The experiment was conducted on individual leaves of the trees that had been used in the tree experiment (see next section); the leaves had an average surface area of 140 cm^2.

Larvae may react to (fresh) larval tracks; however, there is no clear evidence for the persistence of these tracks (Marks 1977; Meisner et al. 2011). Moreover, Moser et al. (2010) postulate that larval tracks play only a minor role in foraging behaviour of *H. axyridis*. For adult female ladybirds the persistence of the oviposition-deterrent effect of larval tracks has been shown to be 5 to 10 days (Hemptinne et al. 2001; Ruzicka 2002). Therefore, individual leaves were used at least 5 days after the tree experiment had been conducted, to allow the larval tracks to diminish.

For each observation, two larvae were gently placed on the surface of a horizontally positioned leaf that had not been used before in the leaf experiment. After the second larva was placed on the leaf, the behaviour of the two individuals was recorded continuously with The Observer XT 10.0. An observation was ended when: 1) one larva left the leaf by dropping from the leaf; 2) one larva walked off the leaf onto the branch (henceforward referred to as "leaving"); 3) one larva attacked, caught, and preyed upon the other larva; or 4) after 1000 seconds, when none of the other three options occurred (henceforward referred to as 'time-out'). We used a behavioural sequence adapted from Yasuda et al. (2001) (figure 1). A distinction was made between actor (= the acting individual) and reactor (= the responding individual). A counter contact or counter attack resulted in a change in actor and reactor. Behaviour after contact was divided into aggressive responses by the actor (attack, catch, predation), and non-aggressive responses by the reactor (no reaction, runaway, drop). Runaway and drop were considered escape responses. During some observations multiple contacts were made; these were treated as independent events in the statistical analysis if the time between two consecutive contacts was more than ten seconds. Each day that the experiment was conducted, at least one replicate of each treatment (species combination) was tested. Replicates and treatments were executed randomly. The number of replicates per treatment and the total number of replicates depended on the number of larva available and on logistic constraints.

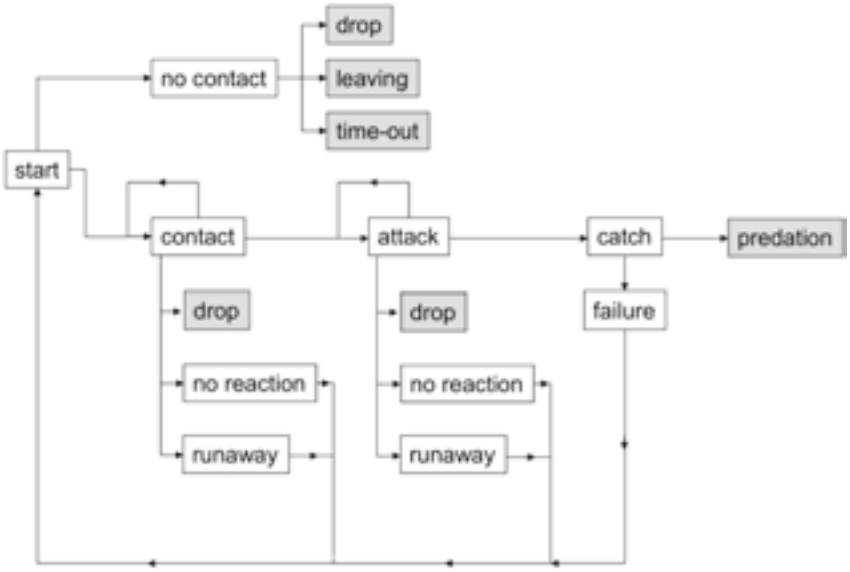

Figure 1 *Behavioural sequence of coccinellid larval encounters. Grey shaded behaviour indicates end of the observation (adapted from Yasuda et al. 2001).*

Tree experiment

In this experiment the spatial scale was increased even more than in the leaf experiment, as the larvae were observed on whole trees: interactions were probably not only affected by escape rates but also by encounter rates. Two-year-old lime trees (*T. platyphyllos*) in 10 litre containers were used. Each tree was pruned to one 50 cm stem and watered daily. On a table (85 cm x 560 cm) six consecutive arenas (85 cm x 85 cm) were constructed from stiff, black plastic boarding (50 cm high), allowing us to test the six species combinations simultaneously. The arenas were filled with a 30 cm layer of white sand. Tanglefoot at the inner side of the plastic boarding prevented the larvae from escaping. On the day of the experiment one container with one tree (with on average eight branches and 53 leaves) was placed in each arena. The container was buried in sand, creating a flat surface stretching from the boarding to the tree stem (see appendix). Two larvae were placed on the upper sides of two leaves at two opposite sides of the tree, on average 50 cm from each other. The position of the larvae on the tree and their behaviour were recorded every five minutes for three hours. Table 1 provides the definitions of the behavioural categories. The experiment was repeated 15 times, resulting in fifteen 3-hour observations of each of the six different larval combinations, summing up to a total of 90 observations. Larval combinations were rotated between the different arenas so that spatial differences could be accounted for. All observations started approximately at 11:30 am and were recorded with The Observer XT 10.0 (Noldus Information Technology B.V., Wageningen, The Netherlands). After 24 hours, the fate of the larvae (alive or dead) and their position was recorded.

Table 1 *Description of larval coccinellid behaviour*

Behaviour	Actor or reactor	Description
contact	actor	larvae touch each other with any body part
attack	actor	larva attacks other larva
catch	actor	larva catches other larva
predation	actor	larva preys upon other larva
counter contact	reactor	larva reacts to contact with new contact
drop	reactor/end experiment	one of the larvae drops from leaf
no reaction	reactor	larva does not change behaviour after contact or attack
counter attack	reactor	larva reacts to attack with new attack
failure	reactor	larva struggles itself free after being caught
runaway	reactor	larva runs away after contact, faster than walking speed
leaving	end experiment	one of the larvae walks over petiole onto branch
time-out	end experiment	1000 seconds have passed without the experiment ending by drop, leaving, or predation

The actor is the acting individual; the reactor is the responding individual

Data analysis

In a large number of the observations during the leaf experiment, the larvae did not make contact with one another, resulting in a many censored data. Survival analysis is then an appropriate technique to analyse the time from the start of an observation

until the first contact, since this approach incorporates the time until a certain event occurs and includes censored data (chapter 2). An event is defined as an *a priori*-defined incident that happens to an individual. In this study an event was the contact between larvae, and censored data were situations where the experiment was ended before the event had occurred (e.g. time-out, leaving without contact, or drop without contact). Data were plotted with Kaplan-Meier's product limit estimator. The Log-rank test was used to test whether covariates had a significant effect on the time until contact for the different species (combinations). As the survivor curves of the species do not necessarily have a similar form (see figure 2) these differences cannot be analysed with Cox's proportional hazards (chapter 2). The effect of temperature on the time until contact was analysed with Cox's proportional hazards model.

To compare attack, catch, and predation frequencies between the different species, contingency tables were constructed and analysed with Pearson χ^2 test. For the leaf experiment each contact was considered an independent event. Results are presented using frequencies, which were calculated as follows: attack frequency was calculated as percentage of the number of contacts, and predation frequency was calculated as percentage of the number of attacks. For the tree experiment, the number of 3-hour observations in which two larvae were on the same leaf at least once was compared between treatments. In some 3-hour observations, the two larvae were observed on one leaf multiple times. These were treated as independent incidents in the subsequent statistical analysis for contact frequency because these

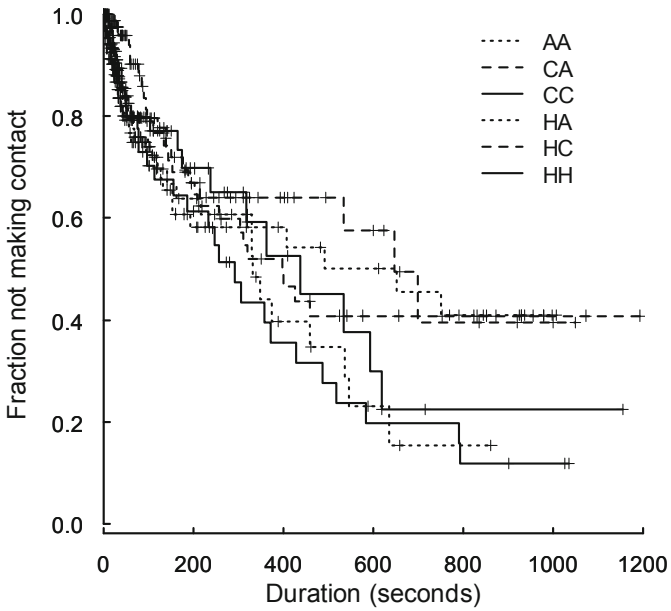

Figure 2 *Effect of species combination on time until first contact. Survival curves of difference in time until contact between fourth instar larvae per species combination during 1000-second observations. Censored observations are marked with '+'. Abbreviations: H* = H. axyridis*, C* = C. septempunctata*, and A* = A. bipunctata*.*

occurred as separate incidents during the three hours. Contact frequency was calculated as the proportion of times a contact occurred when two individuals were present on one leaf; attack and predation frequencies were calculated similar to the leaf experiment. When the expected frequencies were too low to detect deviations from a discrete uniform distribution, Fisher's exact test was used. All statistical analyses were performed with PASW Statistics (18.0.3, 9 Sept 2010). When multiple tests were performed on the same dataset, the critical p-value was Bonferroni-corrected.

Results

Leaf experiment

The leaf experiment was performed 416 times. The total number of observations on each date varied between 6 and 66 (mean: 19). The number of replicates per treatment is given in table 2. In 23–43% of the observations the larvae made contact (table 2). The time until contact differed significantly between combinations when all contacts were pooled (Log-Rank test, pooled pairwise comparisons, P = 0.008) (figure 2). However, when we distinguished between the first contact between two larvae and all later contacts between those two larvae, time until the first contact did not differ between combinations (Log-rank test, pooled pairwise comparisons, P= 0.088) while the time until a second or later contact did differ between combinations (Log-rank test, pooled pairwise comparisons, P = 0.007).

Table 2 *Results of observations in leaf experiment*

	All observations				**Contact observations**			
	Number of observations		**Contact frequency**	**Total number of contacts**	**Acting species**	**Attack**	**Catch**	**Predation**
Treatment	without contact	with contact	(% of observations)			(% of contact)	(% of attack)	(% of catch)
HH	37	28	43%	59	H	14%	0%	0%
CC	54	17	24%	33	C	15%	0%	0%
AA	39	20	34%	41	A	10%	0%	0%
HC	52	29	36%	38	H	24%	78%	86%
					C	5%	0%	0%
HA	42	24	36%	33	H	27%	100%	44%
					A	6%	0%	0%
CA	57	17	23%	23	C	9%	100%	100%
					A	0%	0%	0%

Attack, catch, and predation frequencies are presented for the observed contacts per treatment (= species combination). Total number of replicates per treatment is the sum of the columns 'without contact' and 'with contact'. During one observation, more than one contact can be made. Abbreviations: H = Harmonia axyridis*, C =* Coccinella septempunctata*, and A =* Adalia bipunctata*.*

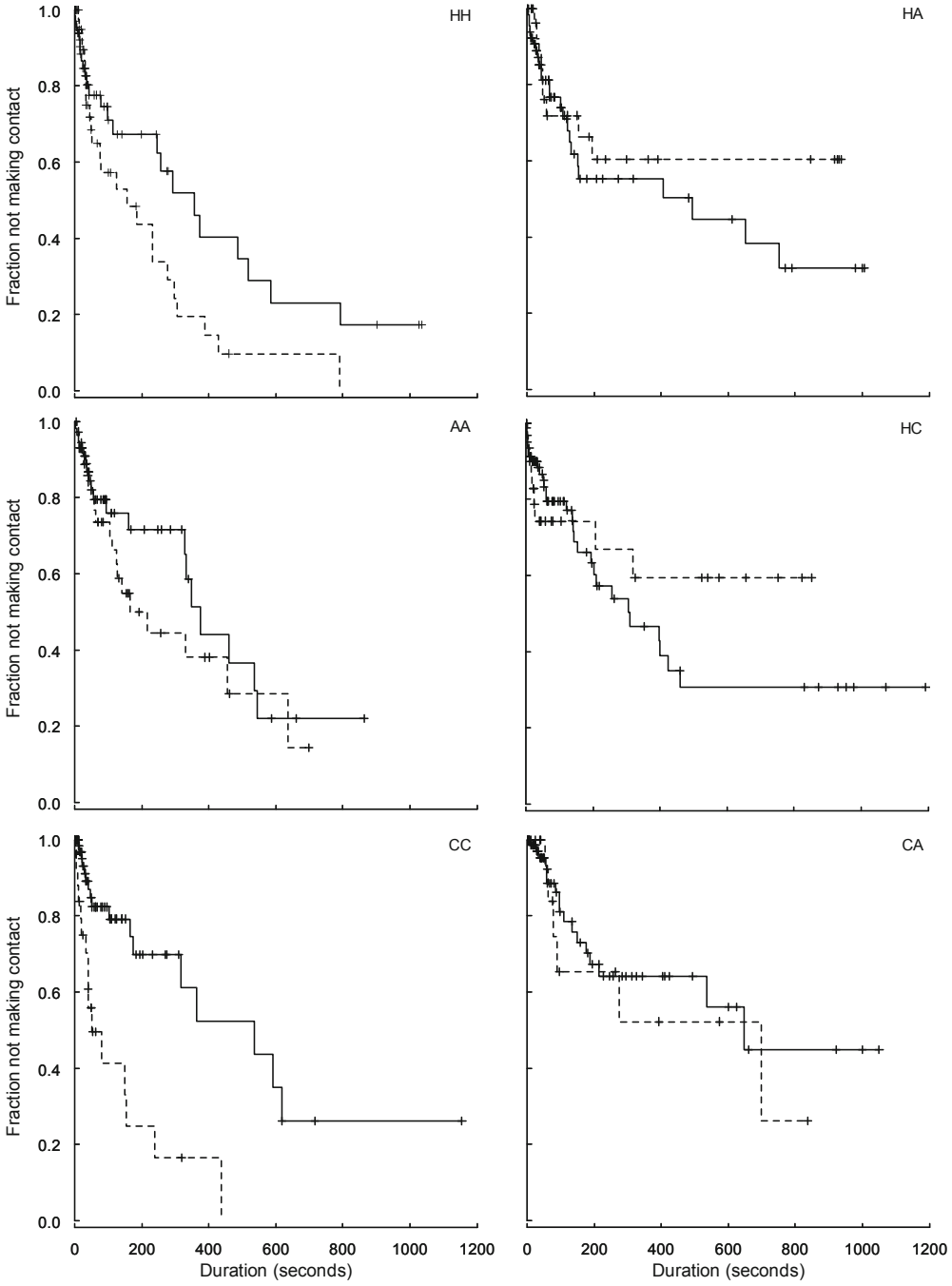

Figure 3 *Effect of previous contact on time until contact. Survival curves of differences in time until first (solid line) and second or later contact (dashed line) are shown for each species combination. Censored observations are marked with '+'. Abbreviations: H* = Harmonia axyridis*, C* = Coccinella septempunctata*, and A* = Adalia bipunctata*.*

Further analysis per treatment showed that the time until subsequent contact differed from the time until first contact (Log-rank test, pooled pairwise comparisons, P = 0.018). Figure 3 shows that for the combinations of *H. axyridis* and heterospecifics, the time until the second or later contact was longer than the time until first contact, while for other combinations the time until subsequent contact was shorter than the time to initial contact. We also tested whether time to first contact was influenced by temperature. When temperature was high (≥ 25°C), the time until the first contact was 3.3 times shorter than when temperature was low (< 25°C) (figure 4, Cox' regression model, Wald test = 13.521, df = 1, P ≪ 0.001).

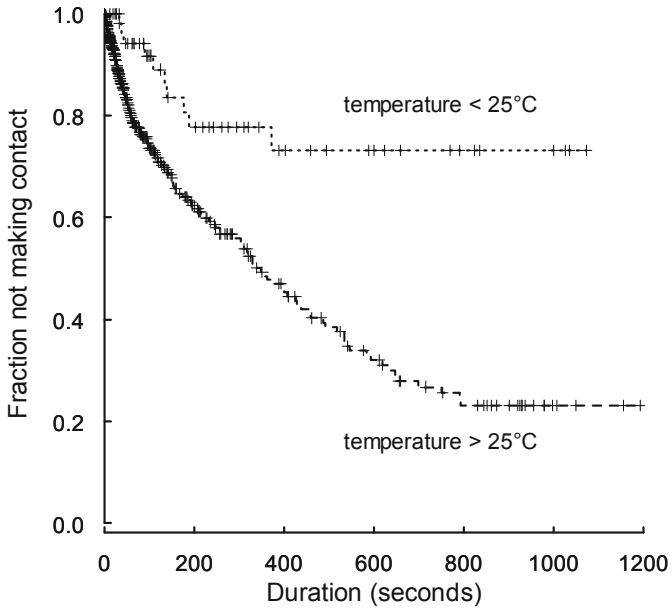

Figure 4 *Effect of temperature on time until first contact. Survival curves of differences in time until first contact at temperatures below and above 25°C are shown for each species combination. Censored observations are marked with '+'.*

When no contact was made, the observation could end in three ways: drop, leaving, or time-out. *Coccinella septempunctata* dropped earlier and more often than the other two species (Log-rank test, P ≪ 0.001). The time until a larva left the leaf did not differ between species (Log-Rank test, pairwise comparisons, P > 0.647) and only 11 observations ended in time-out. This number was too low to analyse the effect of species on time-out statistically.

Aggressive responses

The number of contacts per treatment and the proportion leading to attack by either of the two larvae are shown in figure 5A. There was no difference in attack frequency between species, irrespective of the treatment (χ^2_2= 3.450, Pearson P = 0.178).

However, when the species combination was taken into account, *H. axyridis* tended to attack heterospecifics more often than conspecifics (χ^2_1 = 2.801, P = 0.094), while *A. bipunctata* and *C. septempunctata* attacked heterospecifics as often as conspecifics (χ^2_1= 1.560, Fisher's exact test P = 0.205 and χ^2_1 = 1.827, Fisher's exact test P = 0.162 respectively, figure 5C). In addition, *H. axyridis* caught heterospecifics more often than it caught conspecifics (figure 5C, χ^2_1 = 6.081, Fisher's exact test P = 0.013). This outcome could not be analysed for the other species due to the low number of catches. Both catches by *C. septempunctata* on heterospecifics (*A. bipunctata*) resulted in predation.

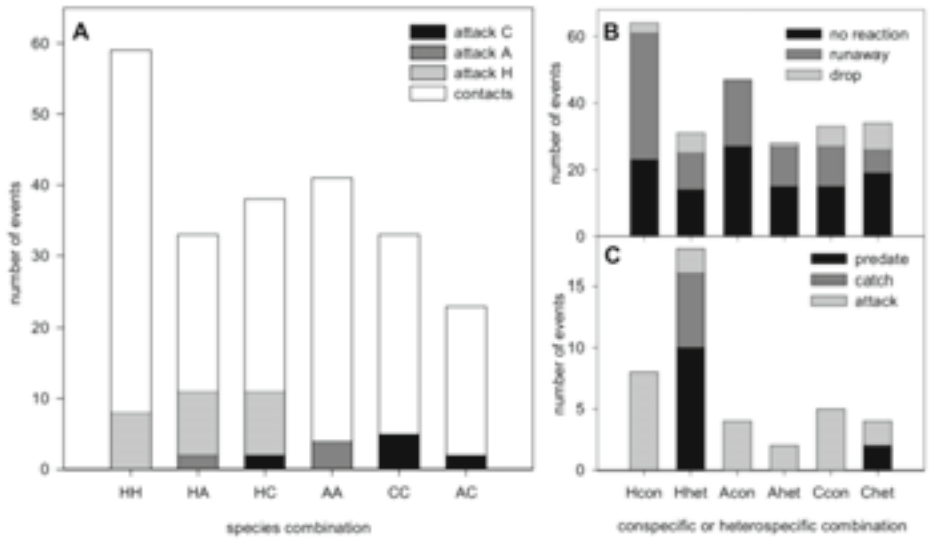

Figure 5 *Behaviours observed in leaf experiments. Two fourth instar larvae were observed for 1000 seconds on one leaf. A: Total number of contacts made per species combination during all observations. Total number of observations per species is given in table 2. B: Total number of non-aggressive responses after contact, when paired with conspecifics or heterospecifics (abbreviated to 'con' and 'het' respectively). C: Total number of aggressive responses after contact, when paired with conspecifics or heterospecifics (abbreviated to 'con' and 'het' respectively). Abbreviations*: H = Harmonia axyridis, C = Coccinella septempunctata, *and* A = Adalia bipunctata.

Non-aggressive responses

The number of non-aggressive responses after contact is shown in figure 5B. Pairwise χ^2-tests, not accounting for treatment, showed that *C. septempunctata* dropped after contact more often than *A. bipunctata* (χ^2_1 = 12.677, Pearson P < 0.001) and that *H. axyridis* ran away after contact more often than *C. septempunctata* (χ^2_1 = 7.884, Pearson P = 0.005). The number of larvae showing no reaction after contact was similar for all three species (χ^2_2 = 5.416, Pearson P = 0.067). The dropping frequency of all three species was not influenced by the treatment (Fisher's exact test: χ^2_1 = 0.567, P = 0.510; χ^2_1 = 0.740, P = 1.000; and χ^2_1 = 0.434, P = 0.552 for *H. axyridis, A. bipunctata,* and *C. septempunctata* respectively). In contrast, treatment did influence runaway

frequency. For all three species the runaway frequency was higher in pairings with conspecifics than in pairings with heterospecifics (Pearson: $P < 0.001$, $P = 0.005$, and $P = 0.004$ for *H. axyridis, A. bipunctata,* and *C. septempunctata* respectively). The frequency of larvae showing no reaction was only influenced by the treatment in case of *H. axyridis* and *A. bipunctata* and was higher in pairings with conspecifics (Pearson: $P = 0.015$ and $P < 0.001$ for *H. axyridis* and *A. bipunctata*, respectively).

Tree experiment

Location and behaviour was recorded at 540 time points (36 time intervals for 15 replicates) for each of the six treatments (N = 3,240 time points). Two larvae on one leaf were observed only one to ten times per treatment (figure 6), and this did not show a significant association with treatment ($\chi^2_5 = 6.274$, Fisher's exact test $P = 0.246$). In seven out of 90 (15 replicates multiplied by six treatments) 3-hour observations two larvae were observed on one leaf more than once. Contact occurred 12

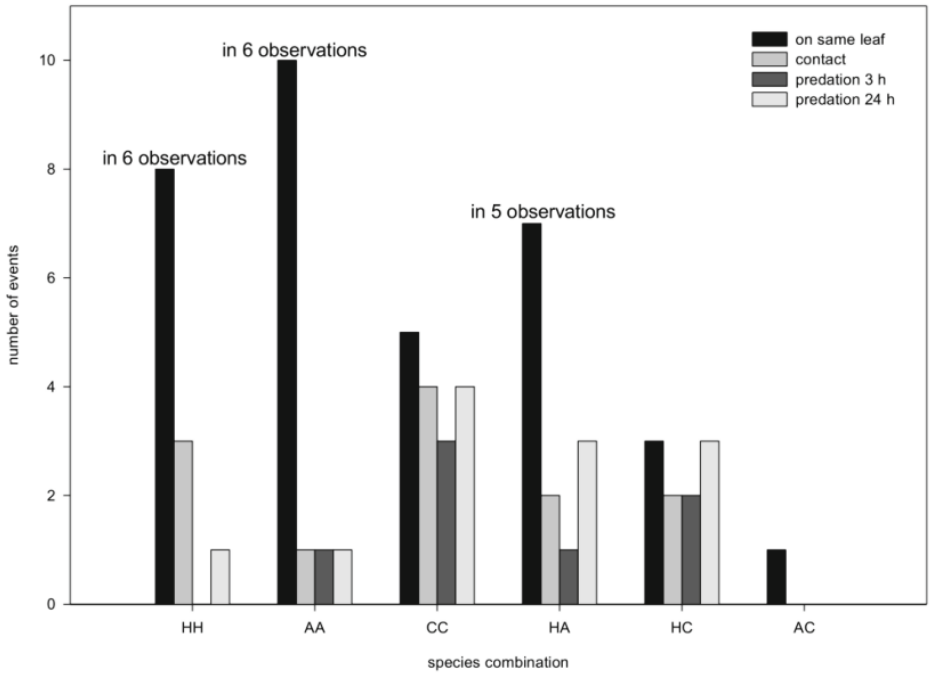

Figure 6 *Incidents observed in tree experiment. Total number of incidents per species combination of two fourth instar larvae observed on a lime tree during 540 time points (all data summed over 36 time intervals of 15 3-hour observations). The number of observations is indicated for the three treatments where the larvae were observed on the same leaf more than once. Abbreviations*: H = Harmonia axyridis, C = Coccinella septempunctata, *and* A = Adalia bipunctata. *Predation in the combination HA and HC was always by* H. axyridis.

times in total; contact frequency (as percentage of being on same leaf) was not significantly associated with treatment (χ^2_5 = 9.171, Fisher's exact test P = 0.084).
Seven of the 12 contacts resulted in predation, again this was independent of species combination (χ^2_4 = 6.857, Fisher's exact test P = 0.169). After 24 hours the number of predation incidents had almost doubled to 12. Differences in predation according to species could not be further analysed due to the low number of incidents, the large differences in dropping frequency between species and the low survival of larvae after 24 hours on the sand. Survival of *C. septempunctata*, in particular, was low (14 larvae out of 60 survived, with only 5 still on the tree). *Coccinella septempunctata* larvae dropped more often from the tree than larvae of the other two species (figure 7, χ^2_2 = 89.902, Pearson P < 0.001). Sometimes, the larvae (of all three species) were able to return to the tree. Interestingly, this happened more often when it was cloudy than when it was sunny (figure 3, tested for *C. septempunctata* χ^2_1 = 8.400, Pearson P = 0.004).

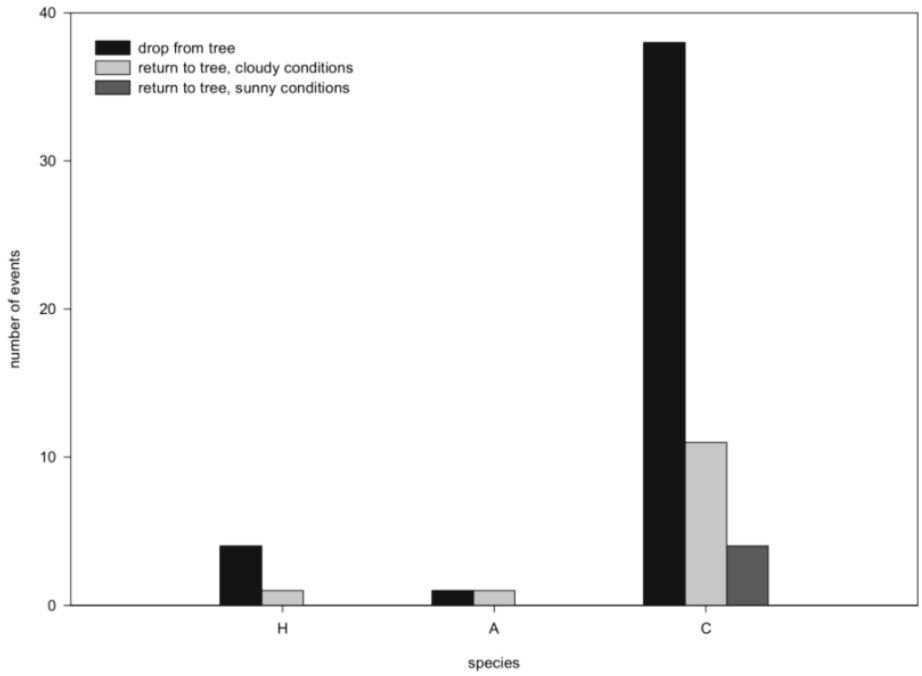

Figure 7 *Larvae dropping from lime tree. Total number of incidents (dropping from and returning to lime tree) by fourth instar larvae during 540 time points (all data summed over 36 time intervals during 15 3-hour observations per species combination). Abbreviations*: H = Harmonia axyridis, C = Coccinella septempunctata, *and* A = Adalia bipunctata.

Discussion

Contact between larvae

Our results clearly show some remarkable differences from those of laboratory studies. The results of previous laboratory experiments showed higher contact frequencies than were observed in our study (e.g. Sato et al. 2009). Second, in laboratory experiments contacts between larvae result in behaviours that differ from those observed in the field due to limited escape possibilities. Generally, escape behaviours, such as fleeing, dropping from the plant, or retreating in refugia are important defensive mechanisms used by insects in the field (Lucas 2005; Pell et al. 2008). Therefore, it is difficult to predict field IGP-frequencies on the basis of laboratory results (Weber and Lundgren 2009).

In most of our observations on individual leaves, one of the two larvae dropped or walked off the leaf before making contact (table 2, figure 4). Our observations suggest that if two larvae made contact, it was purely based on chance. No specific search pattern or type of prey recognition based on vision or olfaction was observed and, although larvae are able to perceive tracks of other larvae (Meisner et al. 2011), they apparently only perceived the other larva when they touched them. Indeed, most studies report that ladybird larvae do not seem to perceive their prey before touching it (Kesten 1969; Wratten 1973; Kawai 1976; Ferran and Dixon 1993; Hodek and Honek 1996 and references therein), or only react to alarm pheromone of their (crushed) prey over short distance (Stubbs 1980; Hemptinne et al. 2000). Visual information seems to be unimportant in prey-searching behaviour (JL Hemptinne pers. comm.), and olfactory cues have only been shown as contact pheromone between adult males and females (Hemptinne et al. 1996). However, the potential role of chemical communication between coccinellid larvae and their prey deserves more attention in future studies.

The likelihood of two larvae making contact was increased by the similar walking pattern of the three species. Our larvae showed a preference for walking along a vein or along the edge of the leaf, which is in line with the findings of earlier studies (e.g. Wratten 1973; Kawai 1976; Ferran and Deconchat 1992). In field conditions, this behaviour increases the chance of encountering aphids, as the density of lime aphids is higher near the veins (Wratten 1973).

The assumption of random contact is also supported by our observation that the time until first contact was similar for all species combinations. After first contact the two larvae are already near each other on the leaf, increasing the chance that they come into contact again, which explains why the time until second contact is generally shorter. Moreover, after the first contact between heterospecifics, *H. axyridis* predates regularly (4 out of 18 contacts with *A. bipunctata* and 7 out of 24 with *C. septempunctata*), while *H. axyridis* and *A. bipunctata* paired with conspecifics had second and later contacts in 50% of the cases. Predation excludes the possibility of a second contact and this explains the differences in time until second contact between treatments. During the 3-hour observations, all predation incidents were on the tree, and after 24 hours larvae that predated on the other larva were always found on the tree, while the larvae that did not survive were all found on the sand. This is a strong indication that scavenging on dead larvae did not happen during our experiment. The

survival of larvae after 24 hours on the sand was low, in particular for *C. septempunctata* (only 14 larvae out of 60 survived, with 5 still on the tree). This low survival was probably caused by the dry and warm weather conditions.

In small arena experiments in the laboratory, contacts between two individuals occur often (Sato et al. 2009), while in most of the 90 observations of the tree experiment which more closely reflected field conditions, the two larvae were never observed on the same leaf. The larvae spent most time walking, in some cases up and down the whole tree. The (very) low number of observations of two larvae on the same leaf was probably caused by the 3-dimensional architecture of the habitat and by the size of the tree. The more complex habitat structure of trees − with branches, leaves, and possible refugia − is an important factor to consider when extrapolating results from laboratory to field (Weber and Lundgren 2009).

Response to contact

Despite the low number of contacts observed in these experiments, when the outcome of all contacts is analysed, predation behaviour by *H. axyridis* was found to be quite high. *Harmonia axyridis* had more catches and successful predations than the other two species, and it mainly attacked and preyed upon heterospecifics (figure 2 and 7B). Overall, *H. axyridis* won in most encounters. The order of predation success is: *H. axyridis* > *C. septempunctata* > *A. bipunctata.* So, *H. axyridis* appears to be the most aggressive of the three species. The superiority of *H. axyridis* as an intraguild predator has also been reported in laboratory experiments with *A. bipunctata, C. septempunctata* (table 3) (Yasuda et al. 2001; Hautier 2003; Sato et al. 2009), and several other coccinellid species (Yasuda et al. 2004; Ware and Majerus 2008; Katsanis et al. 2010).

Several studies using molecular methods confirm that IGP occurs in the field (Hautier et al. 2008; Gagnon et al. 2011; Hautier et al. 2011; Thomas et al. 2013): *H. axyridis* collected at various sites contained 0–53% exogenous alkaloids from *A. bipunctata, Adalia decempunctata* L. (Coleoptera: Coccinellidae)*, Calvia spp., Coccinella septempunctata, Coleomegilla maculata* De Geer (Coleoptera: Coccinellidae)*,* and *Propylea quatuordecimpunctata* L. (Coleoptera: Coccinellidae). Interestingly, *H. axyridis* also appeared as prey in *C. septempunctata, C. maculata,* and *P. quatuordecimpunctata*. These studies reported high levels of IGP, which is not surprising, as our observations were short compared with the duration of a full development cycle from egg to adult emergence. When considering the full lifespan, even a low contact and predation frequency could amount to an overall high number of contacts and predation incidents. Moreover, on our experimental tree with an average total branch length of 300 cm, a density of two larvae is low. During the aphid peak, wild larval densities may be ten to twenty times higher than in our experimental setup (PW de Jong pers. obs. and pers. comm.). In addition, model simulations have shown that even very low frequencies of encounters and predation events may lead to large differences in fitness in favour of the intraguild predator. Thus, IGP might play a substantial role in the invasion success of *H. axyridis* (Kindlmann 2010). With longer durations of possible contact the total probability of contact occurring will increase. After 24 hours the number of predation incidents had increased from 7 (after 3 hours)

to 12. So, despite the low number of contacts observed, predation was recorded in 12 out of 90 observations.

Table 3 *Attack, catch, and predation frequencies in literature*

Combination	Acting species	Attack	Catch	Predation	Reference
		(% of contact)	(% of attack)	(% of catch)	
HH	H	40%	5%		Yasuda et al. 2001
CC	C	5%	5%		Yasuda et al. 2001
HC	H	55%	55%		Yasuda et al. 2001
HC	C	15%	0%	0%	Yasuda et al. 2001
HA	H	40%	87%	76%	Hautier 2003
HA	A	8%	85%	0%	Hautier 2003

Attack, catch, and predation frequencies are presented per species per species combination. Catch rates have been calculated using published escape rates Abbreviations: H = Harmonia axyridis*, C =* Coccinella septempunctata*, and A =* Adalia bipunctata*.*

The multiple defence lines of *H. axyridis* (size (Ware and Majerus 2008); chemical deterrence (Sloggett et al. 2011), and morphological structure (spines) (Hautier et al. 2010)) combined with its aggressive attack behaviour might explain the higher IGP-frequencies of *H. axyridis* against heterospecifics as compared to conspecifics. As *H. axyridis* established in Europe recently, defences of native ladybird species against predation by *H. axyridis* have not yet co-evolved (Ware and Majerus 2008).

Results from earlier laboratory experiments and from our semi-field experiments are in line with knowledge of the defence mechanisms used by ladybirds: *A. bipunctata* is the weakest species since its chemical defence is not effective when paired with *H. axyridis* (Sato et al. 2005), and *C. septempunctata* can protect itself reasonably well as its general dropping behaviour seems to be effective when paired with *H. axyridis*. Remarkably, after being caught by *H. axyridis, A. bipunctata* managed to escape from a catch by *H. axyridis* in five out of nine incidents in our study, whereas *C. septempunctata* managed to escape after being caught by *H. axyridis* only once in seven incidents. Yasuda et al. (2001) also reports lower escape rates of *C. septempunctata* larvae from attacks by *H. axyridis.* Fourth instar larvae of *A. bipunctata* are equally able to escape an attack by *H. axyridis* as *H. axyridis* is able to escape an attack by *A. bipunctata,* whereas younger instars are less successful in escaping (Hautier 2003).

Species combination did not influence dropping frequency after contact. The other two non-aggressive responses (runaway and no reaction), occurred more often in pairings with conspecifics than with heterospecifics. This lower frequency of non-aggressive responses in heterospecific pairings might indicate that for all species – not only for *H. axyridis* as we have shown – the frequency of aggressive responses is higher in heterospecific pairings, but due to low numbers of observations we could not statistically test for differences.

Dropping behaviour

In both experiments, larvae of *C. septempunctata* often dropped from the leaf, corroborating results of Sato et al. (2005). When dropping occurred after contact, we considered it escape behaviour, but some individuals also fell without any apparent cause. Sato et al. (2003) observed that *C. septempunctata* emigrates from the plant sooner than other species when aphid density decreases, thus reducing the occurrence of IGP and cannibalism. It is not clear whether this emigration was caused by larvae dropping from the plant or walking off the plant. *Harmonia axyridis* and *A. bipunctata* prefer trees and shrubs (Hodek and Honek 1996; Sato et al. 2005). For the arboreal species *A. bipunctata* the risk of falling is low as the larvae have a large anal disc for holding onto leaves (Hodek 1973; Carter et al. 1984; Dixon 2000). We observed that *H. axyridis* larvae were also capable of holding onto the leaf with their anal disc, as has been suggested by Osawa (2011). *Coccinella septempunctata* prefers herbaceous vegetation over trees as host plant (Ferran and Dixon 1993; Hodek and Honek 1996) which may explain their frequent dropping behaviour: it is easy to return to the host plant when vegetation grows close to the ground. Further, *C. septempunctata* is able to forage for aphids on the ground, and this ground foraging is estimated to provide them with 30% of the daily diet in wheat (Ferran et al. 1991).

The results of this study show that *H. axyridis* wins in most encounters with heterospecifics and is the strongest intraguild predator of the species tested here. Being an invasive species in Europe and North America, the strong IGP-pressure of *H. axyridis* potentially affects the balance between this invasive predator and native intraguild predators within the aphidophagous guild (cf. Hall 2011). This may result in reduced diversity of native coccinellids in this guild, but does not have to result in changes in aphid densities. There are reports of improved control of pest aphids after release and establishment of *H. axyridis*, but whether non-damaging aphid densities are more likely to increase or decrease following changes in coccinellid diversity is not yet clear (Crowder and Snyder 2010). Most IGP studies, including those conducted at (semi-) field level, are conducted on the level of individual interactions. Although studies in semi-field conditions give more realistic results than laboratory-based experiments, extrapolation to community and ecosystem level is needed to fully understand the effects of an invasive species on aphidophagous guild and its ecosystem service of aphid suppression. Large field community experiments, in combination with modelling studies such as those described by (Shea and Chesson 2002; Hall 2011) might provide this understanding.

Acknowledgements

Thanks to JG de Boer and EJH Dekker for their assistance in using Observer, to G Mekking and A Siepel for technical assistance, and to L Hemerik for statistical advice. We thank the following persons for various forms of assistance: PAC Apeldoorn, SW Benedictus-van den Berg, CAJM van Boxel, P Copini, JMG Haarhuis, K Helling, A Hiscox, Mr & Mrs Huijsman, PW de Jong, MJ Raak, JM Stam.

Appendix chapter 6

Six testing arenas with lime tree. Eache arena was filled with white sand creating a flat surface stretching from the boarding to the tree stem.

7

Invasive alien species under attack: natural enemies of *Harmonia axyridis* in the Netherlands

BioControl (2014) 59: 229-240

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Abstract

The aphid predator *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) is an invasive alien species in Europe and North America with negative effects on non-target species (including a decline of native ladybird populations), as well as fruit production, and human health. It is, therefore, important to find out which natural enemies could be used to reduce their numbers. Knowledge of *H. axyridis'* natural enemies is summarised and data collected from the Netherlands over the past ten years are presented. Beetles were sampled from winter aggregations and from spring through to autumn with illuminated screens at night. Natural enemies were not found in samples of *H. axyridis* from 2003–2007. From 2008 onward *H. axyridis* adults were infested by: *Hesperomyces virescens* Thaxt. fungi (summer and winter); *Parasitylenchus bifurcatus* Poinar and Steenberg nematodes (winter); *Coccipolipus hippodamiae* (McDaniel and Morrill) mites (winter); and *Dinocampus coccinellae* (Schrank) parasitoids (summer and winter). Our results indicate that these natural enemies are starting to use *H. axyridis* as a host, but are as yet not sufficiently abundant to control the population.

Introduction

The multicoloured Asian ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) is an invasive alien species in Europe and North America with negative effects on non-target insect species, fruit production, and human health (Koch and Galvan 2008; Van Lenteren et al. 2008). Moreover, a decline in native ladybird populations is associated with its establishment and rapid spread (Roy et al. 2012). *Harmonia axyridis*, an efficient predator of aphids, is native to northeast Asia (Brown et al. 2011b). It was first introduced into North America as a biological control agent in 1916, followed by numerous releases at many locations through the 1980s (Gordon 1985), but first became an established species in 1988 in the state of Louisiana, USA (Krafsur et al. 1997). In northwest Europe *H. axyridis* was first introduced in 1982, it was used for aphid control in greenhouse crops from 1990, and it was released in the Netherlands in outdoor crops in 1996. In the Netherlands the first specimen (a pupa) was reported from the wild in October 2002 (Cuppen et al. 2004b), and the population of *H. axyridis* has rapidly increased in size and range since then.

Increased understanding of the invasion process and knowledge of mechanisms that promote alien species in becoming established and invasive is needed to limit the effect of these species on native communities (Roy et al. 2011a). In addition to life history traits that promote invasion, an alien species introduced to a new range may experience reduced impacts from natural enemies because it invaded without its native natural enemies, and/or because natural enemies in the invaded area are not (yet) effective in attacking the invader (Enemy Release Hypothesis, Roy et al. 2011a). Many parasitoids, parasites, and pathogens are reported to attack ladybirds, and under certain circumstances these may cause important ladybird mortality (Ceryngier et al. 2012).

Knowledge about natural enemies of *H. axyridis* in the native and invaded range is currently very limited and more detailed studies are needed to draw conclusions on, and predict, the effect of natural enemies on population dynamics of *H. axyridis*. Roy et al. (2011b) provided an overview of natural enemies of coccinellids and discussed potential novel interactions between *H. axyridis* and natural enemies. In the appendix, we provide an extensive survey of the current knowledge of natural enemies attacking adult *H. axyridis*: biology, ecology, and prevalence of entomopathogenic fungi *Hesperomyces virescens* Thaxt. (Ascomycota: Laboulbeniomycetes: Laboulbeniales), nematodes *Parasitylenchus bifurcatus* Poinar and Steenberg (Tylenchida, Hexatylina: Iontonchioidea, Parasitylenchidae), ectoparasitic mites *Coccipolipus hippodamiae* (McDaniel and Morrill) (Acarina: Podapolipidae), and insect parasitoids *Dinocampus coccinellae* (Schrank) (Hymenoptera: Braconidae).

Here, we document natural enemies that attack *H. axyridis* in the Netherlands, describe the infection rates over the past decade, and discuss the potential effects of these natural enemies on *H. axyridis* populations. This study serves as a baseline for monitoring the prevalence of natural enemies of *H. axyridis* over time in the Netherlands and surrounding areas. It may also be used to evaluate strategies to control this invasive organism.

Materials and Methods

Sampling Harmonia axyridis

Winter sampling

Over the course of five successive winters (2006–2010), aggregations of hibernating *H. axyridis* were collected (n = 378, 878, 13521, 19085, and 3105 respectively) at various locations and on various dates in the Netherlands. Beetles were also sampled during their migratory flights (2004, 'De Kaaistoep' (n = 333) and 2010, Wageningen (n = 185)). For details see appendix table A1.

Ladybirds sampled in 2008, 2009, and 2010 were transported at ambient temperature to Wageningen. The beetles were allowed to continue overwintering in outdoor cages behind wooden shutters on the wall of an experimental farm of Wageningen University (N 51 59 32, E 5 39 43) as described earlier (chapter 3). Over the course of the winter, samples of beetles were taken from the overwintering cages and then either directly frozen or first used in experiments and frozen afterwards (chapters 2 and 3). Beetles sampled in 2004, 2006, and 2007 were immediately stored in ethanol (70%).

Summer sampling

Since 1995 insects have been monitored regularly, at night, from March to November (hereafter referred to as summer) each year, in 'De Kaaistoep', near Tilburg (N 51 32 25, E 5 0 53). Insects, attracted to a vertically-placed, white, illuminated, polyester screen (3.5 m wide, 1.9 m high), were sampled with an aspirator after they had landed on the screen, as described in Van Wielink and Spijkers (2013). Observations started at sunset and continued for four hours.

Sampling efforts were not constant over the years. In mid-July 2003, the first *H. axyridis* adult was caught. As a result of this observation and to monitor the arrival of *H. axyridis* sampling effort was intensified in the years 2004–2006. In the period from 2003 to 2011 insects were observed during 361 nights and *H. axyridis* was caught during 208 nights (n = 6216 specimens). No *H. axyridis* were caught in the months March and November in any year. All *H. axyridis* individuals were collected, killed with ethylacetate and stored in ethanol (70%).

Prevalence of natural enemies

Laboulbeniales

All ladybirds collected during summer 2003–2011, during migratory flight in 2004 and 2010, during winter 2006, 2007, and 2010 and a subset of beetles collected during winter 2008 and 2009 were checked for visible infection with Laboulbeniales. During winter 2010 beetles were still alive during examination. Ladybirds without visible infection were used in experiments for two to four weeks at warm experimental conditions (25°C) until shortly after first oviposition and were subsequently frozen. Beetle couples that reproduced late, or not at all, were kept longer at warm conditions until death or late oviposition. Voucher specimens were identified by D Haelewaters and are deposited at Farlow Herbarium, Harvard University (FH). DNA was extracted (Weir and Blackwell 2001) from seven thalli from one host (# D Haelew. 113, female from Tilburg, winter 2010) and the sequence of small subunit ribosomal DNA

(SSU rDNA) was established (methods in Hansen et al. 2005) with primer set NS1/NS4 (White et al. 1990) (GenBank KC833471).

Parasitoids, mites, and nematodes

To determine infection with mites, nematodes and parasitoids, the frozen or ethanol-stored beetles were inspected under the elytra for mites, and the abdomen was dissected to detect nematodes and parasitoids. Beetle sex and colour were recorded. Female ladybirds collected during summer (2003–2011) as well as (sub) samples of beetles (both sexes) collected during winter (2006–2010) were examined (appendix A1).

Nematodes from the different locations were examined by G Karssen with light microscopy and compared with Danish *P. bifurcatus* specimens provided by GO Poinar (Poinar and Steenberg 2012). DNA was extracted from five nematode specimens, collected in Alkmaar, Deelen-K, Tilburg, and Winssen (winter 2010). After amplification of nearly full length SSU rDNA, fragments were cloned and sequenced as described in Holterman et al. (2006) (GenBank KC875397, KC875398, KC875399, KC875400, KC875401). Following alignment of these new sequences in a phylum-wide framework harbouring about 2,700 SSU rDNA sequences (Vervoort et al. 2012), a local phylogenetic tree was constructed, including members of the suborder Hexatylina only.

When mites were found, the elytrae with the mites were stored in 70% ethanol, and specimens were sent to RW Husband for identification.

Parasitoid larvae found during dissection and parasitoid larvae emerging from adult ladybirds during the experiments of 2008 and 2010 were collected. For identification the CO1 gene of two parasitoids, collected in Tilburg and Kootwijk (winter 2010), was sequenced (Folmer et al. 1994) (GenBank KF021264) and compared with that of *D. coccinellae* specimens that had earlier been identified morphologically by C van Achterberg.

Statistical analysis

Statistical analyses were performed separately for winter and summer data, as data collection strategies were different. During winter, aggregations of hibernating *H. axyridis* were collected at different locations in the Netherlands, but not all locations were visited each year. During summer, *H. axyridis* was collected at a single location over the years 2003–2011. Sampling efforts were neither constant over years nor months.

Statistical analysis consisted of fitting generalised linear models (GLM., McCullagh and Nelder 1989) (procedure GENMOD of SAS/STAT® software, version 9.2 (SAS Institute Inc., 2008)), see table 1. We allowed for interactions between explanatory variables, if possible, but removed these from the final models if not significant ($P > 0.05$). Regression coefficients (rc) in the logistic regression models were interpreted in terms of odds ratios (OR, where OR is reported as exp(rc)). Likelihood ratio test statistics χ^2 were used, unless mentioned otherwise.

Table 1 *Overview tested relationships, datasets, response and explanatory variables. H. vir codes for* Hesperomyces virescens

Part	Dataset	Response variable	GLM specification	Explanatory variables	Period
1	all	fraction *H. vir* infected	binomial (n > 1), logit link	location, sex	2010
2a	dissected	*H. vir* infected (y,n)	binomial (n = 1), logit link	location, sex, colour, nematode	2010
2b		nematode infected (y,n)	binomial (n = 1), logit link	year, location, sex, colour, *H. vir*	2008–2010
2c	experiment[a]	female non-fertile (y,n)	binomial (n = 1), logit link	location, colour, nematode, *H. vir*	2010
3	summer	fraction *H. vir* infected	binomial (n > 1), logit link[b]	year, sex, season (Jun–Sep)	2008–2011

[a] *excluding six females: (non-)fertility could not be determined (chapter 3)*
[b] *excluding individuals with unknown sex*

Results

No fungi, nematodes, mites or wasps were found attacking *H. axyridis* in 2003–2007. In 2008, 2009, and 2010, several natural enemies were found. Results are presented below for each natural enemy.

Hesperomyces virescens

Before 2010 no *H. virescens* infection was found in winter samples described in the present study. In another study (chapter 4), however, five (out of 753) beetles sampled in winter 2009 were infected with Laboulbeniales but these were not identified to species level. During the winter of 2010, a remarkably larger number of infected beetles was found. Figure 1 shows the counts, with infection rate ranging from 0% (Deelen-K and Winssen) to 55.6% (Winterswijk). When analysing the 2010 infection rates (table 1, part 1), we found significant differences between locations ($P < 0.0001$, $\chi^2_{11} = 671.6$) and sex ($P < 0.0001$, $\chi^2_1 = 38.6$). Male ladybirds had 1.73 (= exp(rc) = exp(0.55), SE(rc) = 0.09)) greater odds of infection than female ladybirds.

From six locations all beetles (or a representative sample) were dissected individually (appendix A1, n = 1472); 348 beetles were infected with *H. virescens*, 89 with nematodes, and 23 with both. Statistical analysis (table 1, part 2a) showed significant differences in *H. virescens*-infection rates between the locations ($P < 0.0001$, $\chi^2_5 = 289.5$). Tilburg had the highest infection rate of those six locations. Again, males had higher infection rates than females ($P < 0.0001$, $\chi^2_1 = 20.4$; OR = 1.87 (= exp(0.63), SE(rc) = 0.14)). An association of fungal infection with nematode infection was found for location Tilburg ($P = 0.0009$, Wald $\chi^2_1 = 11.0$), where nematode-infected beetles had 4.9 (= exp(1.60), SE(rc) = 0.48)) times greater odds (for infection with *H. virescens*) than non-nematode-infected beetles. In other locations, *H. virescens*-infection rates were low and were not significantly associated with nematode infection ($P = 0.67$, Wald $\chi^2_1 = 0.18$). No relationship of *H. virescens* infection with ladybird colour was found ($P = 0.94$, $\chi^2_2 = 0.12$).

A partial SSU rDNA sequence (728 bp) was obtained, which perfectly matches two known SSU rDNA sequences of *H. virescens* (GenBank AF298233, JQ941711).

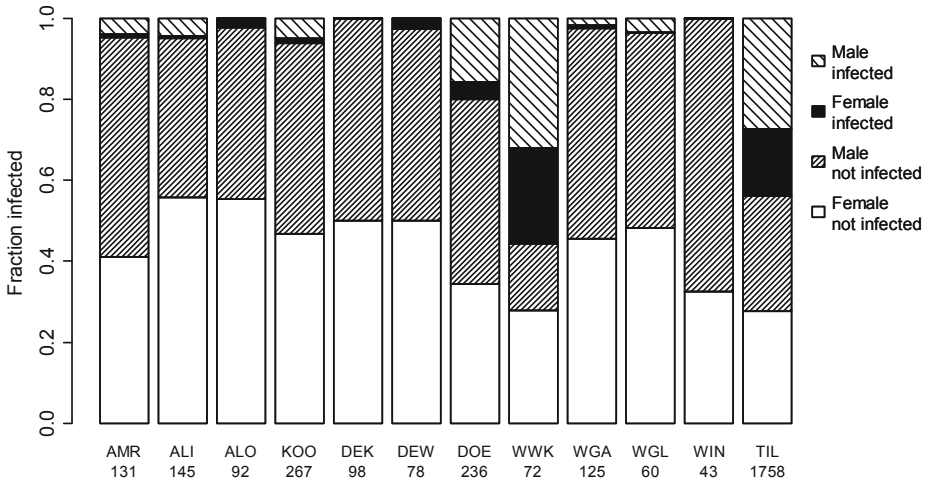

Figure 1 *Prevalence of* Hesperomyces virescens *on* Harmonia axyridis *adults, sampled from winter aggregations in 2010. The X-axis shows the sampling locations and number of beetles collected. Prevalence was recorded by means of visual inspection. Abbreviations for locations are given in appendix table A1.*

In the summer samples the first infection with *H. virescens* was recorded in July 2008. During the years 2008–2011, *H. axyridis* beetles were collected from 56 of 111 observation nights. On 18 nights ladybirds were infected with *H. virescens* (n = 65, table 2), 63 of which could be used in the analysis, as sex was unknown for two adults (July 2008 and July 2009). Analysis of the infection rate (table 1, part 3) showed significant differences between years ($P < 0.0001$, $\chi^2_3 = 55.7$). No significant association with season ($P = 0.81$, $\chi^2_1 = 0.057$) or sex ($P = 0.44$, $\chi^2_1 = 0.58$) was found.

Table 2 *Prevalence of* Hesperomyces virescens *on* Harmonia axyridis *adults, collected with an illuminated screen in 'De Kaaistoep' during spring-autumn in the years 2008–2011*

Year[a]	Season[b]	Observation nights (N)	Total collected (N)	*H. virescens* infected (N (%))
2008	spring	21	42	0 (0)
	summer	14	1088	5 (0.5)
	autumn	0	0	-
2009	spring	14	1	0 (0)
	summer	20	699	27 (3.9)
	autumn	2	0	-
2010	spring	6	0	-
	summer	13	242	18 (7.4)
	autumn	2	7	0 (0)
2011	spring	5	5	1 (20)
	summer	10	339	14 (4.1)
	autumn	4	8	0 (0)

[a] *see also Haelewaters et al. 2012 (period 2008–2010)*
[b] *spring: March–May, summer: June–September, autumn: October–November*

Coccipolipus hippodamiae

Ten ladybirds were found to be infected with Podapolipus mites. Mites on three specimens were positively identified as *C. hippodamiae* by RW Husband. One infected voucher specimen has been deposited at the Museum of Zoology of the University of Michigan, USA. The mites were found on ladybirds sampled during the winters of 2009 (Kootwijk (1) and Zundert (2)) and 2010 (Alkmaar (2), Alphen-O (1), Kootwijk (1), Tilburg (3)). Prevalence ranged from 0.38% (Kootwijk 2010) to 2.6% (Zundert). Both male and female ladybirds were infected. Two male ladybirds from Tilburg were infected with both mites and *H. virescens.* No co-infection with nematodes was observed. No mites were found in the summer samples.

Parasitylenchus bifurcatus

In the winter samples from 2008, 2009, and 2010, nematode infection was found in ladybirds from all locations studied, except Zundert. Additionally, in 2010, incomplete samples from six other locations were dissected (appendix A1), namely only the few beetles that died during the experiment. In five cases at least one of the dissected beetles was nematode infected (Deelen-K, Deelen-W, Winssen, Wageningen-A, and Wageningen-L), indicating that the prevalence was at least 5%. In 2008, beetles from four locations (Alphen-O, Deelen-K, Deelen-W, Loenen-O, n = 2097) overwintered together in one cage: four of the 52 dissected beetles were found infected. Thus, nematode infection has been reported in almost all populations. Both juveniles, males and adult females, were found in one ladybird. Determination based on morphological characteristics showed that these nematodes were identical to the nematodes found in Denmark and, therefore, belong to the species *P. bifurcatus* (Poinar and Steenberg 2012).

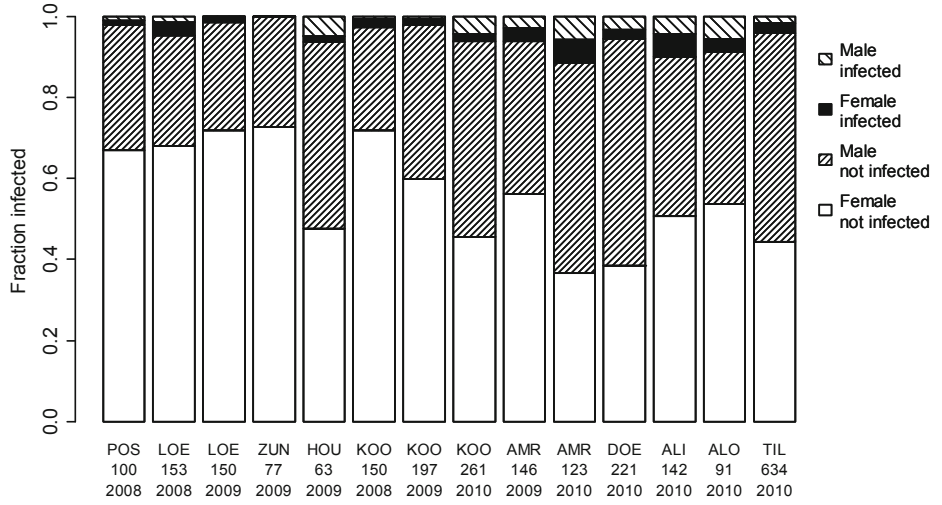

Figure 2 *Prevalence of* Parasitylenchus bifurcatus *in* Harmonia axyridis *adults collected from winter aggregations in 2008, 2009, and 2010. The X-axis shows the sampling year, sampling location, and the number of beetles dissected. Abbreviations for locations are given in appendix table A1.*

The nearly complete SSU rDNA sequences (appr. 1700 bp) of the nematodes originating from the different locations were identical and did not match any known SSU rDNA sequence. The sequences represented a well-supported separate group within the suborder Hexatylina constituting a part of a cluster which also includes *Fergusobia* and *Deladenus*. Hence, ribosomal DNA sequence data show that the species found belongs to a separate genus; *Parasitylenchus*, which is in line with the morphological identification of this species.

Analysis (table 1, part 2b) showed that infection rates differed between years (P = 0.010, χ^2_2 = 9.2) and locations (P = 0.00026, χ^2_9 = 32.1) (figure 2). Neither sex (P = 0.94, χ^2_1 = 0.0062) nor colour (P = 0.51, χ^2_2 = 1.33) was significantly linked to nematode infection rate. In Tilburg, nematode infection showed a positive relationship with *H. virescens* infection (P = 0.002, Wald χ^2_1 = 9.68; OR = 4.40 = exp(1.48), SE(rc) = 0.48)). This relationship was not found at the other locations (P = 0.71, Wald χ^2_1 = 0.14).

During winter experiments in 2010, 5.4% of the females (from locations where all ladybirds were dissected, n = 478) failed to reproduce. Interestingly, 65.4% of the non-reproducing females were infected with nematodes, compared to 2.7% of the reproducing females. Analysis (table 1, part 2c) showed that failure to reproduce was neither significantly related to location (P = 0.60, χ^2_5 = 3.63), nor to colour (P = 0.12, χ^2_2 = 4.17). The association with nematode infection was very strong (P < 0.0001, χ^2_1 = 76.7; OR = 121 (= exp(4.80), SE(rc) = 0.64)). Reproduction was negatively associated with *H. virescens* infection (P = 0.012, χ^2_1 = 6.36). In summer no nematodes were found.

Dinocampus coccinellae

During the winter of 2009, one braconid larva was found by dissection in a ladybird from Zundert representing 0.2% of all dissected beetles in 2009 and 1.3% of dissected beetles from Zundert. In winter 2010, two parasitoids were found: one braconid larva by dissection in a ladybird from Tilburg (0.1%), and one braconid larva emerged from a ladybird from Kootwijk and spun a cocoon (0.004%). No other larvae emerged from the ladybirds during experiments in 2008 and 2010. The sequence of the CO1 gene of both wasps confirmed that the two larvae from 2010 (Tilburg and Kootwijk) belonged to the species *D. coccinellae*. Unfortunately, the larva from Zundert was lost during processing.

Of the summer samples, two parasitoids were found: one braconid larva – probably *D. coccinellae* by dissection (0.4% of 245 dissected females in 2009) – and one *D. coccinellae* larva emerged from a ladybird (0.3% of 352 caught adults in 2011). This specimen was identified by R Comont and H Disney. Both male and female ladybirds were infected.

Discussion

Six years after the first record of *H. axyridis* in the Netherlands in 2002 (Cuppen et al. 2004b), we first observed attacks by natural enemies (fungi and nematodes). Here we show that, although the immune system of *H. axyridis* protects against most fungi, nematodes, bacteria, and yeast (Gross et al. 2010), three species, *H. virescens*, *P. bifurcatus*, and *C. hippodamiae*, are able to overcome the immune system of *H. axyridis*.

Hesperomyces virescens

Hesperomyces virescens was found parasitising *H. axyridis* for the first time during the summer of 2008 in samples collected from 'De Kaaistoep'. In overwintering aggregations, the first *H. axyridis* found infected with Laboulbeniales was recorded in 2009 in Zundert, which is close to 'De Kaaistoep'. The variation in prevalence which we observed between years as well as between locations is also reflected in other studies (appendix A2, A3, and Haelewaters et al. 2012). For comparison, *H. virescens* infection rates on *H. axyridis* in North America varied both over season (Riddick 2006) and between sampling sites (Nalepa and Weir 2007). Successful establishment of *H. virescens* requires both a suitable host and favourable environmental conditions, as experimentally shown for Carabidae (De Kesel 1996a), which may explain the variability in infection rates. Moreover, apparent absence of infection (e.g. on *A. bipunctata* in France and Switzerland, appendix A3) can be attributed to a combination of low sampling intensity and (very) low parasite prevalence (Welch et al. 2001).

In our study, host sex was significantly linked to infection rate in winter populations (more males than females were infected) but not in summer populations. In other studies the relationship between sex and infection is not consistent: some observe differences in infection rate (summer, Garcés and Williams 2004; winter, Nalepa and Weir 2007) while others do not (fall or winter, Riddick and Schaefer 2005; summer, Riddick 2006). Host sex is also associated with thallus distribution over the body (e.g. Riddick 2006; Nalepa and Weir 2007), and with thallus density (Riddick and Cottrell 2010). Differences between sexes in infection rate, thallus distribution and density are suggested to be caused by different (mating) activity (Riddick and Schaefer 2005; De Kesel 2011), as active beetles have a higher chance of coming into contact with fungal spores than inactive beetles.

Infection rates in Doesburg, Tilburg, Winterswijk, and those observed in Germany in 2009 (Herz and Kleespies 2012) are almost as high as the infection rates in overwintering populations in the USA (appendix A2). Infection rates in Belgium have increased from 2007 to 2011 (De Kesel 2011) in line with summer infection rates reported here. This may imply that *H. virescens* is spreading through the *H. axyridis* population in Europe, resulting in higher infection rates, confirming the suggestion by Roy et al. (2011b).

Winter prevalence of *H. virescens* in Tilburg, 2010 (which is near "De Kaaistoep") is much higher than in the summer (2010 and 2011). Several factors influence infection levels; Firstly "dilution" of the proportion of infected beetles following the emergence of a new, uninfected, cohort of adults during the summer could explain this difference (Welch et al. 2001). Secondly, a substantial part (20%; range 0–65%) of the beetles caught in summer had soft elytra, indicating that that these beetles had only recently emerged and are therefore uninfected, as it takes three to four days for the exoskeleton to sclerotise and darken (Obata 1988). Finally, summer observation nights were not equally distributed over the months. This is partly due to the fact that *H. axyridis* only flies when the following requirements are met: a) temperatures not far below 14°C; b) no rain, and; c) wind not too strong (PS van Wielink pers. obs.), i.e. conditions that are not easily met in spring and autumn.

The observed negative effect of *H. virescens* infection on reproduction in our study can be explained by the fact that beetles that did not reproduce were kept at warm conditions in the experiment for much longer than the other beetles. Hence, spores had time to develop into thalli that were detectable during visual inspection.

Based on the available data we are limited to speculating about the origin of the *H. virescens* infection in Europe. The recent discovery of two *H. virescens*-infected *H. axyridis* individuals from the native range, China, sampled in the 1930s (Haelewaters et al. 2014) suggests that *H. virescens* is a historically global species and that at least one isolate was able to attack *H. axyridis* before it started spreading globally. However, we do not expect that infected beetles from Asia were the source of infection in Europe and the USA. Firstly, *H. virescens* has been reported in both Europe (Santamaría et al. 1991) and North America (Thaxter 1891), long before the arrival of *H. axyridis*. Secondly, *H. axyridis* populations have been in quarantine before introduction as biological control agents (Krafsur et al. 1997; Van Lenteren et al. 2008). Thirdly all populations introduced in the USA until 1980 originated from Japan or the USSR (Gordon 1985), where until now no *H. virescens* has been observed.

Instead we suggest that local strains of *H. virescens* have adapted to *H. axyridis* in both Europe and North America. Cottrell and Riddick (2012) showed with their laboratory experiment of forced confinement of healthy and infected beetles, that it is possible that different isolates of *H. virescens* exist and that these isolates may have a high degree of host specificity. We hypothesise that many isolates of *H. virescens* exist. The record from China shows that *H. virescens* is able to attack *H. axyridis*, but in Europe and the USA no isolate adapted to *H. axyridis* existed. Therefore, local isolates of *H. virescens*, infecting other hosts, had to adapt to *H. axyridis*. This can explain the time lag observed between *H. axyridis* introduction and first observation of *H. virescens* in both Europe (6 years) and North America (10 years), where during a survey on museum collections of *H. axyridis* sampled (from 1992 onwards) the first *H. virescens* infected specimens were observed only in 2002 (appendix A2, SY Zhao pers. comm.). Other possibilities are firstly that recent, undocumented, introductions from China are the source of the *H. virescens* infection in North America and/or Europe. Secondly, that infected beetles from North America have been introduced in Europe, although one would expect a shorter time lag in this case, as the possible *H. virescens* isolate was already adapted to *H. axyridis*, unless this had occurred in 2007 or 2008 and it took only a few years to spread and be detected. Further studies comparing invasion routes of *H. axyridis* with *H. virescens* distribution, can shed light on the invasion process of *H. axyridis* and the interaction with *H. virescens.*

Based on our results we suggest that *H. virescens* appears to have established on *H. axyridis* in the Netherlands. How *H. virescens* will affect population densities of *H. axyridis* is unknown, as the effect of this fungus on other coccinellids is negligible (Riddick et al. 2009). However, *H. axyridis* infected with Laboulbeniales had a slightly lower winter survival than uninfected beetles (Riddick 2010).

Coccipolipus hippodamiae

The first *H. axyridis* infected with *C. hippodamiae* in the Netherlands was found in winter 2009. The observed infection rates were low. Because mite infection negatively

influences fitness (Rhule et al. 2010), the impact on beetle mortality may increase when mite infection rates approach levels that are reported for native *A. bipunctata* (appendix A4).

Coccipolipus hippodamiae mites were reported in Europe before the arrival of *H. axyridis* (appendix A4), hence we assume that they are beginning to use the exotic ladybird as a novel host. Webberley et al. (2006b) did not detect mite infection on *A. bipunctata* in the Netherlands, nor in other populations from northwestern Europe, while moderate to high prevalence was observed in eastern and southern European populations. Mite infection of *H. axyridis* is also low in the Netherlands (this study) compared to eastern Europe (appendix A2). This study might indicate the recent arrival of this parasite in Northwest Europe. It seems likely that the Netherlands constitute the current border of the geographical distribution of the mites.

Parasitylenchus bifurcatus

The first observation of *P. bifurcatus* nematodes was in the winter samples of *H. axyridis* in 2008, which is earlier than the first report of *Parasitylenchus* sp. from Denmark in 2009 (Harding et al. 2011) (appendix A2) and concurrent with the first report of 'Allantonematidae' from Germany in October 2008 (Herz and Kleespies 2012, A Herz pers. comm.). The infection rates are comparable with infection rates found in Germany (Herz and Kleespies 2012) and Denmark in 2009 (Harding et al. 2011) (appendix A2). In our study, infection rates differed significantly between years and locations (but not across sexes). Infection rates also differed significantly between locations in studies in Germany (Pearsons exact χ^2, P = 0.0216 (Herz and Kleespies 2012)) and Denmark (Pearsons χ^2, P = 0.0005 (Harding et al. 2011)).

The specimens we found are morphologically similar to *P. bifurcatus* as described by Poinar and Steenberg (2012). It is unknown how *H. axyridis* acquired this nematode infection. The species P*. bifurcatus* was not reported before 2012, and unfortunately, we cannot rule out that this species is similar to *P. coccinellinae,* as the descriptions by both Iperti and Van Waerebeke (1968) and by Poinar and Steenberg (2012) were not complete. Moreover, no DNA data are available for *P. coccinellinae* specimens to make a comparison with our DNA data.

The original source of infection in Europe could be parasitised individuals introduced from Asia or from North America. No records of nematodes infecting ladybirds are known from Asia, while from North America, infection of *H. axyridis* with 'Allantonematidae' (species unknown) has been reported (Roy et al. 2011b). Poinar and Steenberg (2012) suggest that infection could also be obtained from native European ladybirds. The species *P. coccinellinae* attacks several multivoltine coccinellid species: *Propylea quatuordecimpunctata* (L.), *Oenopia conglobata* (L.)*, A. bipunctata, Hippodamia variegata* (Goeze), and *Harmonia quadripunctata* (Pontopiddian) (Iperti and Waerebeke 1968).

Despite low infection rates, nematode infection has been observed in three successive years and rates were highest in the final year (2010). We therefore conclude that the nematodes have established on *H. axyridis* in the Netherlands. Harding et al. (2011) also report increasing infection rates. Infection can reach moderate levels in natural *H. axyridis* populations, up to 33.3% in Denmark (Harding et al. 2011). The

highly significant relationship between non-fertility of female ladybirds and nematode infection supports the hypothesis that *Parasitylenchus* sp. affects the reproduction of the ladybirds (Harding et al. 2011).The combination of moderate infection rates and reduction of reproductive ability of the host, signals the potential effect of *P. bifurcatus* on populations of *H. axyridis* and suggests the possible use of *P. bifurcatus* as biological control agent of *H. axyridis*.

Dinocampus coccinellae

Infection of *H. axyridis* with *D. coccinellae* in the Netherlands was observed for the first time in 2009. Prevalence in the Netherlands is very low compared with prevalence in other ladybird species (appendix A5) and is even low in contrast with prevalence among *H. axyridis* in other European countries and the USA (appendix A6). Parasitism by *D. coccinellae* currently has only a marginal impact on the population dynamics of *H. axyridis* (Berkvens et al. 2010b). With similar attack rates for both *H. axyridis* and native species and a lower emergence from *H. axyridis*, the latter may act as an egg-sink for *D. coccinellae* (as hypothesized by Hoogendoorn and Heimpel 2002). Such an egg-sink may lead to a decline in *D. coccinellae* populations and result in a decline of infection rates in native coccinellid species (Berkvens et al. 2010b).

Simultaneous infection with two natural enemies

Some ladybirds were infected with both *H. virescens* and mites (0.14%) or nematodes (1.6%). Co-infection has been observed earlier for mites in Austria (Christian 2002) and the USA (Riddick 2010), and for nematodes in Germany (Herz and Kleespies 2012).

Little is known about the effect of co-infection on ladybird fitness. Infection with mites and *H. virescens* results in a lower winter survival than infection with mites or fungus alone (Riddick 2010). We found that in Tilburg nematode and *H. virescens* infection were positively associated but, interestingly, not in other locations. If we assume that nematode-infected beetles are more sensitive to *H. virescens* infection, then a high *H. virescens* pressure as observed in Tilburg should result in a high percentage of infection with *H. virescens* in those beetles that are already infected with nematodes. The reduced total number of living beetles with nematodes in Tilburg indicates that co-infection of nematodes and *H. virescens* might also result in a lower survival. Further experiments are needed to study the effect of co-infection of *H. virescens* and mites or nematodes. If survival is indeed reduced, it might open options for biological control of the invasive ladybird.

Mechanisms of transfer

We have suggested that European populations of *H. virescens, C. hippodamiae* and nematodes, are switching from native coccinellids and are starting to use *H. axyridis* as a new host. All three natural enemies are reported in more than one coccinellid host, indicating that they have a more generalist nature. The biological control literature shows that generalist natural enemies make host switches easier than specialist species. Examples for natural enemies of leaf miners are given in Minkenberg and Van Lenteren (1986). Host shifts have been reported for nematodes (McFrederick and Taylor 2013) and Laboulbeniales (Rossi 2011).

In winter, transfer between species could occur during overwintering. Although overwintering ladybirds usually form monospecific clusters, sometimes a few adults of other species are present in the aggregations (Hodek 2012b). We found *A. bipunctata* and *O. conglobata* adults during sampling of overwintering populations of *H. axyridis* at very low numbers (< 0.2%, chapters 2, 3, and 4). Both species are reported as hosts of *C. hippodamiae* and *P. coccinellinae*, and *A. bipunctata* is also reported as a host of *H. virescens* (appendix A3, A4, Iperti and Waerebeke 1968); therefore these ladybird species could be a possible source of infection of *H. axyridis* populations.

In summer, transfer between species could occur during interspecific mating. Reproductive mature males will mount almost any beetle they find – whether male or female, dead or alive, conspecific or heterospecific – to copulate (Obata 1987; Majerus 1994). Interspecific mating in the field has been observed between *H. axyridis* and *Asioresta* sp. and in a treesome of *H. axyridis* with *C. septempunctata* and *A. bipunctata* (AJM Loomans pers. obs.). Female rejection behaviour reduces the actual number of hybrid matings (Majerus 1994), but mounting alone can already facilitate transfer of natural enemies between individuals.

In this study we report that various types of natural enemies are starting to use *H. axyridis* as a host. We suggest that these enemies have transferred from native coccinellids hosts during overwintering or interspecific mating. Nevertheless, as yet, they are insufficiently effective to control this invasive alien species.

Acknowledgements

We would like to thank the following people and organisations for their various contributions. Morphological identification natural enemies: C van Achterberg (Naturalis Biodiversity Center, the Netherlands), RF Comont (Centre for Ecology and Hydrology, UK), RHL Disney (University of Cambridge, UK), RW Husband (Adrian College, Michigan, USA), G Karssen (Plant Protection Service, the Netherlands), GO Poinar (Oregon State University, USA), and RA Samson and J Houbraken (CBS Fungal Biodiversity Centre, the Netherlands). Molecular assistance: KK Beentjes (Naturalis Biodiversity Center, the Netherlands), KF LoBuglio and DH Pfister (Harvard University, USA), SJJ van den Elsen, PJW Mooijman, and P Verbaarschot (Wageningen University). Laboratory assistance: E van Eekelen, J Stam, and W van Tol (Wageningen University). General assistance: CAJM van Boxel, SW Benedictus-van den Berg, Y Jongema, L Hemerik (Wageningen University), K. Oku, MJ Raak, and SY Zhao (Harvard University, USA). Ladybird collection: Mr & Mrs Brandsma, Mr & Mrs Van den Berg, I Folkertsma, A Hakman, K Helling, Mr & Mrs Huijsman, Mr & Mrs Van der Kamp, J. van Kemenade, Mr & Mrs Klok, K Kots, B Lodeweges, AJM Loomans (Plant Protection Service, the Netherlands), E van Pelt, H Spijkers, H. Schep, T Timmer, and E Voorhout. Review and commenting on earlier version of the manuscript: T E Cottrell (USDA, USA) and A Hiscox (Wageningen University). Monitoring in De Kaaistoep with the illuminated screen was supported with a grant of the Uyttenbogaart-Eliasen Stichting.

Appendix chapter 7

Overview of natural enemies attacking adult *H. axyridis*

Entomopathogenic fungi – Hesperomyces virescens

Hesperomyces virescens Thaxt. (Ascomycota: Laboulbeniomycetes: Laboulbeniales) is an obligate ectoparasite that has been reported to infect adults of several coccinellid species (Santamaría et al. 1991). Laboulbeniales complete their full life cycle on the integument of a living host where no mycelium but instead individual thalli are formed directly from ascospores (Thaxter 1896; Tavares 1985; Santamaría 1998). The development from ascospore into mature thallus at 25°C requires 13 to 26 days depending on the host species (Cottrell and Riddick 2012).

Hesperomyces virescens is one of the few species of the Laboulbeniales that is able to penetrate the insect cuticle and forms a notable haustorium entering the body cavity (Weir and Beakes 1996; Santamaría 2003). The thalli can be formed on any part of the body of the insect (Haelewaters et al. 2012). The consensus is that Laboulbeniales cause little or no harm to their hosts (Scheloske 1969; Benjamin 1971; Majewski 1994; Santamaría 1998). However, some negative fitness consequences have been reported (Benjamin 1971; Riddick 2006). Recently with respect to *H. virescens* on *H. axyridis* it is shown that infection with *H. virescens* reduces mating frequency (Nalepa and Weir 2007), and that overwintering adults (particularly males) have a slightly lower survival when infected with *H. virescens* or when infected with both *H. virescens* and *Coccipolipus hippodamiae* (McDaniel and Morrill) (Acarina: Podapolipidae) than adults without these fungi (Riddick 2010). In Israel in *Chilocorus bipustulatus* L. (Coleoptera: Coccinellidae) populations, *H. virescens* infection rapidly spread and was associated with a sudden decline in beetle numbers (table A3). Moreover, Nalepa & Weir (2007) state that clumps of thalli formed on legs, mouthparts, and antennae may inhibit detection of predators, mates, and food or may impede flight.

The sticky spores of *H. virescens* have a short life span, are exclusively spread by activities of the host, and are not transmitted via substrate or through air over long distances (De Kesel 1996b; Cottrell and Riddick 2012). Non-random infection patterns of thalli on the body of both sexes of the host indicate that the most important type of (direct) transmission occurs during sexual contact in the mating/feeding season (Weir and Beakes 1996; Welch et al. 2001; Garcés and Williams 2004; Riddick and Schaefer 2005; Harwood et al. 2006); infection with Laboulbeniales can therefore be considered as a sexually transmitted disease (Welch et al. 2001). In *H. axyridis*, which overwinters in dense aggregations, *H. virescens* is also socially transmitted; in the course of winter the infection pattern shifts and the infection rates increase, suggesting that in overwintering aggregations transmission through direct physical contact is an important mechanism of spread of this fungus (Riddick and Schaefer 2005; Riddick 2006; Nalepa and Weir 2007). Auto-infection is caused by grooming, resulting in high thallus densities on older hosts (Riddick and Schaefer 2005; Haelewaters et al. 2012). Fungus prevalence can reach high levels but can vary widely between locations and from season to season (table A2).

Table A1 *Sampling locations and dates and sample sizes of aggregated hibernating* Harmonia axyridis *adults in winter 2004–2005, 2006–2007, 2007–2008, 2008–2009, 2009–2010, 2010–2011, the Netherlands*

			Collection year	**2004–2007**		**2008**		**2009**		**2010**	
Location	**Abbr**	**Coordinates**	**Collection site**	**N (dissected)**	**date**	**N (dissected)**	**date**	**N (dissected)**	**Date**	**N (dissected)**	**date**
Alkmaar	AMR	N 52 37 57, E 4 44 35	Urban area, inside unheated church tower on stone wall	-		-		2550 (146)	19 Nov	131 (123)	20 Nov
Alphen a/d Rijn-I	ALO	N 52 7 27, E 4 40 2	Urban area, inside, unheated room	-		547([a])	11 Nov	-		145 (142)	26 Oct
Alphen a/d Rijn-O	ALI	N 52 8 33, E 4 39 22	Urban area, outside, in steel window frames	-		-	-	99	8 Dec	92 (91)	29 Oct 18 Nov
Deelen-K	DEK	N 52 3 53, E 5 53 23	Farmland/woodland border, outside, between stone wall and shutters	-		719 ([a])	7 Nov	623	25 Nov	98 (9[b])	3 Nov
Deelen-W	DEW	N 52 3 37, E 5 53 54	Farmland/woodland border, outside, between stone wall and shutters	-		436 ([a])	7 Nov	434	25 Nov	78 (13[b])	3 Nov
Doesburg	DOE	N 52 0 57, E 6 8 9	Urban area, inside, unheated room	-		-		-		236 (221)	3 Nov
Houten	HOU	N 52 1 39, E 5 9 38	Urban area, inside unheated church tower on stone wall	-		-		5297[c] (63)	27 Nov	-	
Kootwijk	KOO	N 52 10 39, E 5 45 39	Woodland area, outside, between stone wall and shutters; inside, unheated and heated rooms	-		4846 (150)	13 Nov	5166 (197)	13 Nov	267 (261)	8 Oct 15 Oct
De Kaaistoep	KAA	N 51 32 25, E 5 0 53	Farmland/woodland area, on stone wall and white screen, while alighting	333	29 Oct 2004	-		-		-	
Loenen-O	LOO	N 52 7 29, E 6 1 45	Woodland area, inside: partly heated room	-		398 ([a])	13 Nov	-		-	
Loenen-S	LOS	N 52 6 2, E 6 0 23	Woodland area, outside, between stone wall and shutters	-		2530 (153)	13 Nov	1116 (150)	17 Nov	-[d]	
Lunteren	LUN	N 52 5 39, E 5 37 3	Urban area, inside, unheated attic	-		1984	13 Nov	-		-	
Posbank	POS	N 52 1 45, E 6 1 18	Woodland area, outside, in folds of parasols	-		2061 (100)	7 Nov	-		-	
Tilburg	TIL	N 51 33 22, E 5 4 7	Urban area, inside, on stone wall of unheated water tower	378[c] (378) 216[c] (216)	28 Mar 2007[e] 21 Dec 2007	-		-		1758 (756)	30 Nov
Wageningen-A	WGA	N 51 58 53, E 5 40 20	Urban area, outside, on stone wall, while alighting	-		-		-		125 (17[b])	9 Oct
Wageningen-L	WGL	N 51 59 5, E 5 39 49	Urban area, outside, on stone wall, while alighting	-		-		-		60 (16[b])	10 Oct
Winssen	WIN	N 51 53 11, E 5 41 22	Farmland area, inside, unheated room and window frames	-		-		-		43 (2[b])	3 Nov
Winterswijk	WWK	N 51 55 17, E 6 43 8	Farmland/woodland area, inside, unheated room	-		-		-		72 (1[b])	3 Dec
Wouwse Plantage	WPL	N 51 27 38, E 4 23 40	Farmland/woodland border, under unheated roof	662[c]	10 Dec 2007	-		-		-	
Zundert	ZUN	N 51 30 38, E 4 38 36	Woodland area, outside, between stone wall and shutters	-		-		3800 (77)	26 Nov	-[d]	

[a] *Populations overwintered in one cage, 52 individuals of the mixed group were dissected*
[b] *Non-random sample for dissection*
[c] *Part of overwintering populations was collected*
[d] *Low number reported by owners building: no beetles collected*
[e] *These beetles started overwintering in 2006*

Table A2 *Prevalence of* Coccipolipus hippodamiae, Hesperomyces virescens, *and* Parasitylenchidae *with* Harmonia axyridis *as host*

Prevalence	N	Location	Year	Population type	Collection method	Reference
Hesperomyces virescens						
0%	196	USA + Canada	1992–2001		Museum collection study	SY Zhao pers. comm.[g]
31.4%	258	USA, eastern states	2002–2009		Museum collection study	SY Zhao pers. comm.[g]
11–79%(m), 20–27(f)	482+314	USA, Ohio	2002	Summer	Brushing & sweeping	Garcés and Williams 2004
	445	USA, Pennsylvania	2002	Overwintering aggregation (November)		Riddick and Schaefer 2005
52.5%(m), 57.4%(f)	206	USA, Pennsylvania	2003	Overwintering aggregation (March)		Riddick and Schaefer 2005
14%	1740	USA, Pennsylvania	2003	Autumn, overwintering aggregation		Riddick and Schaefer 2005
22–62%	800	USA, North Carolina	2003	Migrating populations & overwintering aggregation		Nalepa and Weir 2007
54.3%(m) 39.4%(f)	436	USA, Mississippi	2003	Summer, early autumn	With nets from trees	Riddick 2006
82.3%	147	USA, Kentucky	2004	Summer	Malaise trap	Harwood et al. 2006
35.6%(m), 40.8%(f)	431	USA, Mississippi	2004	Summer early autumn	With nets from trees	Riddick 2006
20–49%(m), 8–32%(f)	1203	USA, Mississippi	2003	November		Riddick 2006
50.1	646	USA, Georgia	2007	Spring, summer, autumn	Sweeping	Riddick and Cottrell 2010
0.5%	203	Belgium	2006–2007	Overwintering aggregation		De Kesel 2011
2–58%[ac]	1085	Germany	2008–2009	Year round sampling		Herz and Kleespies 2012, A Herz pers. comm.
3.6–43.5%[c]	707	USA, Mississippi	2007–2008, 2008–2009	Overwintering aggregation		Riddick 2010
0.9%	113	Germany	2009	Overwintering aggregation, spring		Steenberg and Harding 2010a
96.5%	86	Belgium	2011	Overwintering aggregation		De Kesel 2011
Coccipolipus hippodamiae						
1.8–17.4%[d]	707	USA, Mississippi	2007–2008, 2008–2009	Overwintering aggregation		Riddick 2010
3.71%	517	Poland	2009	Spring	By eye & beating	Rhule et al. 2010
Parasitylenchidae						
2.3%[ad]	1085	Germany	2008–2009	Year round sampling, autumn 2008–autumn 2009	*Allantonematidae*	Herz and Kleespies 2012, A Herz pers. comm.
2–11.6%[e]	860	Denmark	2009	Late summer, early autumn	*Parasitylenchus* sp.	Harding et al. 2011
90%[e]	500	USA, Minnesota	2009	Summer	*Allantonematidae*	Roy et al. 2011b
10–33.3%	101	Denmark	2010	Early autumn	*Parasitylenchus* sp.	Harding et al. 2011
60%[f]	20	Denmark	2010	November	*P. bifurcatus*	Poinar and Steenberg 2012

[a] *Ladybirds caught during migratory flights were not kept individually before dissection*
[b] *Partly co-infection with Allantonematidae*
[c] *Partly co-infection with* C. hippodamiae
[d] *Partly co-infection with* H. virescens
[e] *Ladybirds were not kept individually before dissection*
[f] *Incubated for 30 days after collection*
[g] *Combined results based on museum collection from various years and origin*

Emergence of a new generation of adults may lower the average level of infection (Riddick 2006; Riddick and Cottrell 2010). De Kesel (2011) suggests that the combination of characteristics of *H. axyridis* (multivoltine, promiscuous, and overwintering in aggregations) aid the spread of *H. virescens*, as these factors all support (intergenerational) contact during the year

Most Laboulbeniales exhibit great host specificity and are restricted to a particular species or genus (Thaxter 1896; Benjamin 1971; Huldén 1983; Majewski 1994; De Kesel 1996a), illustrated by the many published regional parasite-host lists. *Hesperomyces virescens*, however, is reported to infect as many as 20 different coccinellid species (Ceryngier et al. 2012) from around the world since it was first described by Thaxter (1891, as *Stigmatomyces virescens*). *Hesperomyces virescens* was first recorded on *H. axyridis* in Ohio during the summer of 2002 (Garcés and Williams 2004). Since then, infection rates reported for *H. axyridis* have generally been higher than those reported for other coccinellid species (table A3). Infection of *H. axyridis* by *H. virescens* has only recently been reported in northwestern Europe, from Germany (Steenberg and Harding 2010a; Herz and Kleespies 2012), the Netherlands (Haelewaters and De Kesel 2011; Haelewaters et al. 2012), and Belgium (De Kesel 2011). Although Roy et al. (2011b) suggest that *H. virescens* does not attack *H. axyridis* in its native range, recently two Chinese specimens collected in the 1930s were found infested with this parasite (Haelewaters et al. 2014).

Ectoparasitic mites – Coccipolipus hippodamiae

The ectoparasitic mite *Coccipolipus hippodamiae* (McDaniel and Morrill) (Acarina: Podapolipidae) was first discovered on *H. convergens* in the USA and subsequently on *Adalia bipunctata* L. (Coleoptera: Coccinellidae) in the USA and Russia (Ceryngier and Hodek 1996; Riddick 2010). Later, it was reported parasitising *A. bipunctata* and several other coccinellids in Europe (Ceryngier et al. 2012). Recently, *C. hippodamiae* has been discovered to infect *H. axyridis* under natural field conditions in the USA and Poland (table A2).

This mite lives under the elytra of its host and feeds on its haemolymph. Transmission occurs during copulation when motile mite larvae migrate between hosts (Webberley et al. 2004). Adult females have only 2 pairs of legs in adaptation to a parasitic existence (Husband 1984).

Webberley et al. (2006a) report mite prevalence on *A. bipunctata* in Poland (mean: 34%). In overwintering aggregations of *A. bipunctata* infection is low (6.6% in 2000). The number of infective ladybirds (which bear all mite stages, including the motile larvae that can infect other ladybirds) is even lower (2.6% in 2000) and transmission does either not occur or occurs at low rate (Webberley and Hurst 2002). During spring, mite prevalence peaks (up to 90% infection of the ladybirds in 1999 (Webberley et al. 2006a), as transmission occurs at a high rate during mating. When the next susceptible host generation matures, prevalence decreases (Webberley and Hurst 2002; Webberley et al. 2004). Due to host dynamics, prevalence shows several peaks during the season (Webberley et al. 2004). In eastern Europe prevalence on *A. bipunctata* (44.9%) is generally higher than on other native species (table A4). *Coccipolipus hippodamiae* is widely present on *A. bipunctata* in central, southern and

eastern Europe, but absent from populations near the northwestern coast (including the Netherlands) and northern Scandinavia (Webberley et al. 2006b). Webberley et al. (2006b) discuss the correlation between degree of infection and voltinism, as *A. bipunctata* is multivoltine in central, southern, and eastern Europe resulting in reproductive overlap of generations.

Table A3 *Prevalence of* Hesperomyces virescens *on coccinellids other than* Harmonia axyridis *in Europe (and Israel) and North America*

Species	Prevalence	N	Location	Year	Season	Reference
Adalia bipunctata	0%	166	France	1960	Summer	SY Zhao pers. comm.[a]
	0%	50	Switzerland	1975–1989	Spring, summer	SY Zhao pers. comm.[a]
	24%	70	UK	Before 1996	Overwintering aggregation	Weir and Beakes 1996
	0–48%	-	UK	1998	Spring, summer	Welch et al. 2001
	0–50%	-	UK	1999	Spring	Welch et al. 2001
	29%	14	Austria	1999–2001	Overwintering aggregation, summer	Christian 2001
	0%	97	Belgium	2011	Overwintering aggregation	De Kesel 2011
Chilocorus bipustulatus	69.4%	173	Israel	1966	Summer (July)	Kamburov et al. 1967
	95%	-	Israel	1966	Summer (early September)	Kamburov et al. 1967
Brachiancantha quadripunctata	4.2%	48	USA, Kentucky	2004	Summer	Harwood et al. 2006
Cycloneda munda	2.5%	81	USA, Kentucky	2004	Summer	Harwood et al. 2006
Hippodamia convergens	1.3%	297	USA, Georgia	2007	Spring, summer, autumn	Riddick and Cottrell 2010
Hippodamia tredecimpunctata	3.1%	65	USA, Alaska	1944–1978	Spring, summer, autumn	SY Zhao pers. comm.[a]
Olla v-nigrum	33.1%	142	USA, Georgia	2007	Spring, summer, autumn	Riddick and Cottrell 2010
Psyllobora vigintimaculata	4.7%	170	USA, Kentucky	2004	Summer	Harwood et al. 2006
	6.3%	32	USA, Massachusetts	2005	Summer	SY Zhao pers. comm.[a]

[a]*Combined results based on museum collection from various years and origin. Only those species with at least 30 specimens screened were included.*

Infection with *C. hippodamiae* has no significant impact on host survival under laboratory conditions (Hurst et al. 1995), but affects fecundity in *A. bipunctata* (Ryder et al. 2007) and leads to infertility in females of *A. bipunctata, A. decempunctata* L., and *O. conglobata* (L.), after 18.5, 23.5, and 24.5 days post infection, respectively (Webberley et al. 2004). Recently, Rhule et al. (2010) showed that infection also causes infertility in *H. axyridis* females from 19 days after infection onwards. For other Coccipolipus mites, reduction in vigour and fecundity has also been reported (Schroder 1982; Ramaraju and Poorani 2012), although other studies find no effect on fecundity, longevity and feeding (Hochmuth et al. 1987, and references therein).

Table A4 *Prevalence of* Coccipolipus hippodamiae *on coccinellids other than* Harmonia axyridis

Species	Prevalence	N	Location	Year	Season	Reference
Adalia bipunctata	4%	-	Russia	1997	-	Zacharov & Eidelberg (1997) in Christian 2002
	42.4–82.1%(m) 29.1–76%(f)	159 158	Poland	1997	End spring-early summer	Webberley et al. 2006a
	6–92%	> 200 in total	Austria	1999-2001	Spring-early autumn	Christian 2002, E Christian pers. comm.
	10–87%	2497	Poland	1999	Spring- summer	Webberley et al. 2006a
	6.6%	227	Germany	2000	Overwintering aggregation	Webberley and Hurst 2002
	3.5–69.5%	5374	Eastern Europe	1996-2001	Spring-early autumn	Webberley et al. 2004[a]
Adalia decempunctata	0–25.0%	660	Eastern Europe	1999-2001	Spring-summer	Webberley et al. 2004[a]
Calvia quatuor-decimguttata	0–10%	32	Eastern Europe	1996-2001	Spring-summer	Webberley et al. 2004[a]
Oenopia conglobata	0–9.1%	286	Eastern Europe	1996-2001	Spring-early autumn	Webberley et al. 2004[a]

[a]*for detailed prevalence data per location we refer to Webberley et al. 2004*

Overwintering survival is lower for *A. bipunctata* adults infected with *C. hippodamiae* compared to uninfected specimens (Webberley and Hurst 2002). Probably, mites also negatively influence overwintering survival of *H. axyridis* (Riddick 2010). Due to higher mortality of infected beetles, the winter period has a negative effect on mite prevalence (Webberley and Hurst 2002).

Parasitic nematodes – Parasitylenchidae

The nematode *Parasitylenchus coccinellinae* (Tylenchida, Hexatylina: Iontonchioidea, Parasitylenchidae – according to Siddiqi (2000); note that this genus is placed in the family Allantonematidae by several other authors) has first been described by Iperti and Van Waerebeke (1968) as a parasite of several, mostly multivoltine, coccinellid species in southern France. The prevalences differ between coccinellid species: *P. quatuordecimpunctata* (L.) (up to 70%), *O. conglobata* (20%), *A. bipunctata* and *H. variegata* (Goeze) (both less than 10%). The univoltine species *Hippodamia undecimnotata* (Schneider) is only incidentally infected (Iperti and Waerebeke 1968).

Recently, the first *H. axyridis* infected with nematodes were recorded outside their native range. In 2008–2009, representatives of the 'Allantonematidae' were found at low rates in *H. axyridis* in Germany (Herz and Kleespies 2012), and in 2009 at very high rates in Minnesota, USA. In 2009, *H. axyridis* was found infected with a *Parasitylenchus* sp. in Denmark (table A2). This species was described by Poinar and Steenberg (2012) as *Parasitylenchus bifurcatus*.

Members of the genus *Parasitylenchus* are characterised by two amphimictic generations inside their host (Poinar et al. 1997). Mobile fertilised females with a well-developed stylet and apparently activated pharyngeal glands localise and penetrate an insect host. Once it has migrated into the host's hemocoel, the parasitic female starts feeding, swells, and lays eggs. Eggs hatch and the resulting parasitic juveniles develop into secondary adults. The second generation females are morphologically deviant

from the first generation as their body is smaller, and the pharyngeal glands are barely active (hence, females of *Parasitylenchus* show dimorphism: this is not observed for males) (Poinar et al. 1997). These females take up food from the insect host, swell, and produce eggs, which develop into 3rd or 4th stage juveniles that mate inside the host. The secondary vermiform females leave the host, and will not undergo their final moult until penetrating a new host (Ceryngier et al. 2012; Poinar and Steenberg 2012).

Poinar and Steenberg (2012) argue that in contrast to other species of the genus, *P. bifurcatus* can produce multiple generations within one host, whereby the number of generations probably depends on the nutrients available. Large nematode populations can be found in one infected adult ladybird. Iperti and Waerebeke (1968) reported up to 140 adult female nematodes and 10000 nematode juveniles per host. Nematodes are transmitted between adult coccinellids; the larval en pupal stages of the host are not infected (Poinar and Steenberg 2012).

It is not known how infective nematodes localise and enter new hosts (Ceryngier and Hodek 1996; Ceryngier et al. 2012; Poinar and Steenberg 2012). Ceryngier and Hodek (1996) suggest penetration through the sexual organs during mating, through the host tracheae, or through the softer cuticle between the sclerites of the host. Both Ceryngier and Hodek (1996) and Poinar and Steenberg (2012) state that nematode transmission probably occurs in overwintering *H. axyridis* aggregations, particularly under damp conditions, although the highest prevalence is reported in late summer, early autumn (Iperti 1964; Harding et al. 2011).

The effect of nematode infection on ladybird fitness is not known. Depletion of the host's fat body and reproductive organs was reported by Iperti and Waerebeke (1968). Poinar and Steenberg (2012) noted similar effects. They suggest that the impact may be sub-lethal. In *Parasitylenchus*-infected *Drosophila*, females were probably sterile as no mature eggs were detected (Poinar et al. 1997).

Hymenopteran parasitoids – Dinocampus coccinellae

Coccinellids can be parasitised by a number of different hymenopteran and dipteran parasitoids (Ceryngier and Hodek 1996; Ceryngier et al. 2012). Some are known to parasitise *H. axyridis* in its native and/or in its invasive range. Of the three species attacking adults of *H. axyridis*, *Dinocampus coccinellae* (Schrank) (Hymenoptera: Braconidae), *Medina separata* (Meigen) (Diptera: Tachinidae), and *Strongygaster triangulifera* (Loew) (Diptera: Tachinidae), only *D. coccinellae* is reported to attack *H. axyridis* in Europe (Roy et al. 2011b; Ceryngier et al. 2012).

Dinocampus coccinellae (Schrank) is a hymenopteran solitary, koinobiont endo-parasitoid with a cosmopolitan distribution (Ceryngier et al. 2012). In Europe it is has been recorded parasitising adults of at least 18 different species of coccinellids although levels of successful emergence vary highly (Ceryngier et al. 2012). *Dinocampus coccinellae* can attack all life stages except the egg stage, but it prefers, and is most successful in, the adult stage (Roy et al. 2011b). *Harmonia axyridis* is attacked by *D. coccinellae* in its native range, but the parasitism rate of Japanese *H. axyridis* is lower than that of *Coccinella septempunctata brucki* Mulsant (Coleoptera: Coccinellidae) (Maeta 1969). Moreover, parasitoid emergence from *H. axyridis* is

lower, despite parasitoid oviposition at similar frequencies, than from *C. septempunctata brucki* (Koyama and Majerus 2008).

In Europe and North America levels of successful parasitoid development and emergence from adult *H. axyridis* hosts are low (0–17%, table A6) relative to Japan (25% (Koyama and Majerus 2008)) and relative to other coccinellids (table A5).

In laboratory settings, wasps readily attack adults of *H. axyridis* (Hoogendoorn and Heimpel 2002; Koyama and Majerus 2008; Berkvens et al. 2010b) and prefer them over larvae (Firlej et al. 2010), while successful emergence from larvae is higher than from adult *H. axyridis* (Firlej et al. 2007; Berkvens et al. 2010b). Ware et al. (2010) reported successful emergence of *D. coccinellae* larvae from field-collected pupae.

The low emergence rates of the parasitoid are probably due to a lower production of teratocytes (cells involved in immunosuppression and nutrition) by the parasitoid larvae (Firlej et al. 2007) as well as encapsulation of parasitoid eggs by the *H. axyridis* immune system (Firlej and Boivin 2010; Firlej et al. 2012). The poor development of *D. coccinellae* in *H. axyridis* suggests that this parasitoid does not play any significant role in regulation of the host population (Koyama and Majerus 2008; Berkvens et al. 2010b; Roy et al. 2011b).

Table A5 *Ranges of successful parasitism of* Dinocampus coccinellae *in adult coccinellids other than* Harmonia axyridis*. For detailed prevalence rates, host-origin, and references, we refer to Ceryngier et al. (2012), table 8.6.*

Species	Successful parasitism	Origin host
Adalia bipunctata	0–12%	USA
Brachiancatha ursine	4%	USA
Coccinella magnifica	0%	UK
Coccinella septempuncata	64–78%	UK
	32–58%	USA
Coccinella septempunctata brucki	8.9–12%	Japan
Coccinella undecimpunctata	42%	USA
Coleomegilla maculata	18–96%	Northern America
Cycloneda munda	12–57%	USA
Hippodamia convergens	30–92%	USA
Hippodamia parenthesis	72%	USA
Hippodamia variegata	0–17%	Northern America
Olla sp.	39%	USA
Propylea quatuordecimpunctata	1–2%	Northern America

Table A6 *Prevalence and successful emergence rates of* Dinocampus coccinellae *in adult* Harmonia axyridis

Prevalence	Successful emergence	N[a]	Origin host	Year	Population type	Collection method	Identification method	Reference
7.8%	60%/30%[b]	129	Japan	1960	Late spring, pupae	Field collection	Dissection dead beetles; cocoon produced/emergence wasp	Maeta 1969
1.2%	-	164	Japan	1961	Late autumn	Field collection	-	Maeta 1969
-	< 1%		USA	1994	Spring-autumn, adults	-	Emergence wasp	LaMana and Miller 1996
23.8%	-	63	USA	1999	Summer, adults	-	Dissection; cocoons produced	Hoogendoorn and Heimpel 2002
8.9–12%	10%[b]	282	USA	2000	Summer, adults	-	Dissection; cocoons produced	Hoogendoorn and Heimpel 2002
4.6%	0%[b]	942	Canada	2002	Summer, adults	Vacuum random sampling	Dissection; cocoons produced	Firlej et al. 2005
-	3 individuals	-	Belgium	2005	-	Field survey	Emergence wasp	Berkvens et al. 2010b
-	Occasional sightings	-	UK	-	-	-	-	Roy et al. 2011b
5.5%	5%[b]	711	UK	2007	Spring, adults	Field collection	Dissection; emergence wasp	Koyama and Majerus 2008
-	A few individuals	-	Denmark	2006–2009	-	-	Emergence wasp	Steenberg and Harding 2010b
-	2 individuals	-	Denmark	2006–2008	-	-	Cocoons produced	Steenberg and Harding 2009b
3.2%	91%[b]	-	Denmark	2008	-	-	Cocoons produced	Roy et al. 2011b
-	0.8% (0.4%)	1120	UK	2008	Summer, pupae reared into adults	Field collection	Cocoons produced; emergence wasp	Ware et al. 2010
-	4–5%	-	UK	2011–2012	Pupae reared into adults	Field collection	Emergence wasp	RF Comont pers. comm.
Up to 71%	-	-	Denmark	2008	Autumn	-	Dissected	Roy et al. 2011b
1%	-	1085	Germany	2008–2009	Adults, year round sampling	Field collection	Dissected	Herz and Kleespies 2012
-	0–1.7%[c]	60	Belgium	-	Adults, field pop.	Lab parasitisation	Emergence wasp	Berkvens et al. 2010b
-	16.9%[c]	30	Belgium	-	Adults, lab pop.	Lab parasitisation	Emergence wasp	Berkvens et al. 2010b
7%	-	44	USA	-	Adults, lab pop.	Lab parasitisation	Dissection	Hoogendoorn and Heimpel 2002
0%	0%[d]	NA	Canada	-	Adults, lab pop.	Lab parasitisation	Dissection; cocoons produced	Firlej et al. 2007
0%	-	15x3	Canada	-	Adults, lab pop.	Lab parasitisation	Dissection	Firlej et al. 2010
-	7.3%[c]	41	UK	2007	Adults, field pop.	Lab parasitisation	Emergence wasp	Koyama and Majerus 2008
-	25.7%[c]	70	Japan	2007	Adults, field pop. from Japan	Lab parasitisation	Emergence wasp	Koyama and Majerus 2008

[a] *for laboratorium studies number of ladybirds used in the experiment is given*
[b] *emergence relative to number infected*
[c] *emergence relative to number successfully attacked*
[d] *emergence relative to number of host offered*

8

Did the life history of multicoloured Asian ladybird, *Harmonia axyridis*, change when it spread across the globe? A meta-analysis

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Abstract

The multicoloured Asian ladybird *Harmonia axyridis* was introduced to various areas of the world as a biological control agent of aphids. Soon after its introduction negative side effects were reported and it is now considered an invasive alien species. It is still unclear what explains the invasion success of *H. axyridis.* In this study we first investigated whether invasive populations of *H. axyridis* have different life history traits than native Asian populations. A meta-analysis was performed determining the effect of geographic origin, photoperiod, food, ladybird strain, and temperature on rate of development and survival of egg, larval and pupal stages, and the rate of maturation, longevity, and reproduction of *H. axyridis*. The analysis showed that Asian and invasive populations of *H. axyridis* differ in several of their life history characteristics, suggesting that the invasive populations developed from a non-random subset of the beetles in the area of origin, or that evolutionary changes have occurred following invasion. The greatest differences in development rate were observed at temperatures above 24°C, while at a temperature range of 17°C to 24°C, individuals of invasive populations and native Asian populations developed at similar rates. Next, we investigated differences between *H. axyridis* and native European ladybird species. *Harmonia axyridis* develops more slowly than native species and starts reproduction later, suggesting no competitive advantage for the invader. However, it has a higher longevity, fecundity, and number of generations per year, and as a result of this, *H. axyridis* can potentially easily outnumber native species in a few years. In addition, *H. axyridis* is the winner in most intraguild predation interactions with native ladybirds. All these factors combined may explain the invasion success of *H. axyridis*.

Introduction

The multicoloured Asian ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) was introduced to various areas of the world as a biological control agent of aphids. However, soon after its introduction, negative effects were observed, and *H. axyridis* is now considered an invasive alien species in Europe and America (Brown et al. 2011b). Between 1970 and 1990 many experiments were conducted to highlight the biological control capabilities of the ladybird (e.g. Hukusima and Takeda 1975; McClure 1987; Ferran et al. 1996; Trouve et al. 1997). After reports of negative side effects in the late 1990s (e.g. Colunga-Garcia et al. 1997; Brown and Miller 1998; Phoofolo and Obrycki 1998), the focus shifted and studies concentrated on reconstruction of introduction pathways (e.g. Lombaert et al. 2010), non-target effects (e.g. reviewed by Lucas et al. 2007; Koch and Galvan 2008), and explanation of its invasion success (e.g. Evans et al. 2011).

After its introduction, *H. axyridis* established and spread rapidly through America and Europe. It has become the dominant ladybird species in many habitats, and a decline of native aphid predators (both ladybirds and other arthropods) has been reported in North America (Michaud 2002b; Alyokhin and Sewell 2004) and Europe (Brakefield and De Jong 2011; Roy et al. 2012). Besides aphids and coccinellids, various other non-target species have been negatively affected, such as the monarch butterfly, *Danaus plexippus* (L.) and the spined soldier bug, *Podisus maculiventris* (Say) (De Clercq et al. 2003; Koch et al. 2006b). A variety of effects on humans and their crops have been reported. During hibernation, *H. axyridis* adults form large aggregations in cracks and crevices of rocks and trees, but they also aggregate in houses and other buildings, where the ladybirds are a nuisance while walking and flying around, causing an unpleasant odour and stains on carpets, furniture, and walls (Huelsman and Kovach 2004). The (dried) reflex-blood of *H. axyridis* can cause severe allergic reactions in humans (Goetz 2008). In autumn *H. axyridis* adults move into orchards and vineyards, aggregate on the fruit, and switch from carnivory to frugivory (Kögel 2012). The beetles are difficult to remove from harvested fruit and beetles may be crushed during wine processing, causing the wine to smell unpleasant and taste bitter (Kögel et al. 2012b). As a result of these harmful effects, the ladybird is no longer commercially available in most of Europe (Van Lenteren 2012). In its native range in China feeding on date flowers after the wheat harvest has been observed (Li et al. 1992) and temporary invasions into houses during migratory flights (Wang et al. 2011) have been reported.

In Japan the beetle is not regarded as useful natural enemy because it arrives too late for effective aphid control, nor is it regarded as particularly harmful for people, because many beetle species aggregate in houses and no problems with crop damage are known (N Osawa pers. comm.). Aspects of *H. axyridis*, such as biology, morphology, non-target impacts, natural enemies, spread, introduction routes, intraguild predation, and biological control options have been reviewed earlier (Koch 2003; Majerus et al. 2006; Pervez and Omkar 2006; Lucas et al. 2007; Brown et al. 2008a; Kenis et al. 2008; Pell et al. 2008; Poutsma et al. 2008; Brown et al. 2011b; Roy et al. 2011b).

Up to now, however, it has not become clear why *H. axyridis* is such a successful invader. One possible explanation of invasion success is evolutionary change following invasion, due to a genetic bottleneck or selection pressures (Strayer et al. 2006). To

check this possibility, we first conducted a meta-analysis to synthesise evidence collected in a large number of empirical studies that quantified life history characteristics of *H. axyridis* and to generate results that go beyond the results of individual studies that are by necessity limited in scope and size. The question we ask is whether *H. axyridis* has acquired changed life history traits during the invasion, i.e. whether invasive populations differ from native Asian populations, which could possibly explain its success. Empirical studies differed in many respects, e.g. rearing regime of the ladybirds, food, and other conditions of the experiments, such as temperature. Hence, in the meta-analysis we had to account for the effects of (co)variables, most notably temperature, but also day length, whether beetles were fed with aphids or *Ephestia* eggs, and whether they were field collected or reared in the laboratory. Next, we summarised information about life history characteristics of native European ladybird species and compared those with the characteristics of *H. axyridis*, to detect differences and find potential explanations for the invasion success of *H. axyridis*.

Both research questions of this study – 1) can differences between invasive populations and native Asian populations explain the invasion success of *H. axyridis*; and 2) do differences between native European ladybirds and *H. axyridis* provide an explanation for this invasiveness? – will be put into perspective in the discussion.

Methods

Studies from 1900 onwards reporting experimental data on life history characteristics of *H. axyridis* were gathered by literature search in several databases (including CAB abstracts, ISI web of Science, Biological records, Zoological records, and Agricola) and through snowball sampling by checking bibliographies from those papers and previously published reviews. The last search was conducted in July 2013. Search method and selection criteria are more extensively described in the appendix 1. Data were selected from experiments: a) under biologically relevant conditions, excluding extreme photoperiods (< 14 or > 18 hours of light) and temperatures (< 14°C or > 35°C); b) with aphids or *Ephestia kuehniella* Zeller (Lepidoptera, Pyralidae) as prey, excluding data based on toxic or unsuitable food, artificial diet, and other non-aphid food; c) under unlimited food conditions, excluding studies where ladybirds experienced food limitation; d) during summer, excluding studies with post-overwintering populations; and e) for normal, winged beetles, excluding data on beetle strains that cannot fly. Data were also excluded when: a) temperature was not specified; b) temperature was unknown (in case of field studies); and c) deviation from the mean of the sample at the temperature concerned exceeds two standard deviations in that sample (i.e. outliers).

Twenty-two life history characteristics were defined as response variables, representing development and survival of the immature stages that include egg, larval, and pupal stages (further referred to as immature development and immature survival); post-eclosion development; and reproduction. Figure 1 gives an overview of the variables; definitions are given in appendix 1. In the remainder of the text letter-number combinations will be used to refer to the various response variables, these are given at the bottom of each box in figure 1. For immature development and survival

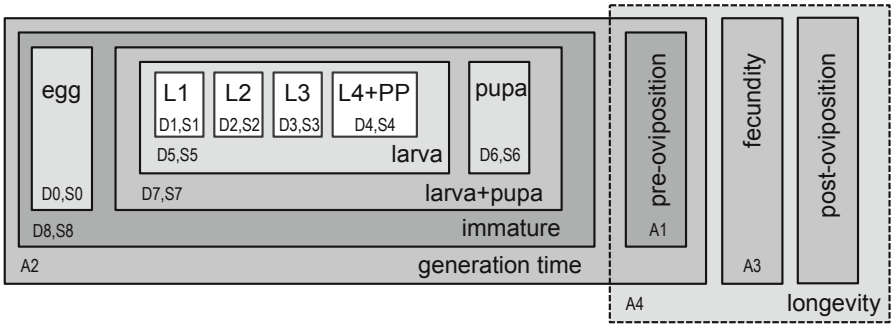


Figure 1 *A schematic overview of the life stages of* H. axyridis *used to define the life history characteristics and their relationships. L1, L2, L3, and L4 refer to four larval instars. The fourth instar includes the pre-pupal stage (PP). Letter-number combinations at the bottom of each box refer to corresponding response variables and are referred to in the text (D0, D1, D2, D3, D4, D5, D6, D7, D8 code for development parameters; S0, S1, S2, S3, S4, S5, S6, S7, S8 code for survival parameters; and A1, A2, A3, A4 code for the parameters for post-eclosion (adult) development).*

the following letters and numbers are used in combination: letters D (immature development) and S (immature survival) and numbers 0 (egg), 1 (L1), 2 (L2), 3 (L3), 4 (L4), 5 (total larval stage), 6 (pupa), 7 (L1 to adult), and 8 (total immature stages). The letter A is used for adult parameters and combined with numbers 1 (pre-oviposition period), 2 (generation time), 3 (daily fecundity), and 4 (longevity). Five explanatory variables were defined: one covariate, temperature, and four categorical variables: geographic origin, photoperiod, food, and strain (table 1). In the analysis of the response variable longevity the factor sex was also included as explanatory variable; data records without information on sex were excluded. Information on food and natural-day was missing for both origins for several survival response variables: in those cases the factor food and/or class natural-day were excluded from the analysis. When the factors origin and photoperiod were confounded, in case of some survival response variables, these were combined into a new factor origin-photoperiod with three levels (table 1).

For each response variable, first the most complex linear mixed model was formulated, i.e. all defined factors were included as long as the effects could be estimated by the statistical programme. Temperature was included as covariate. Each factor could be part of the model as a fixed effect, or additionally in interaction with one of the other factors or with temperature. “Study” is modelled as a random effect, to account for differences between studies that are not accounted for by the categorical variables included in the model. All possible less complex models that could be assembled with the factors of the most complex model, were fitted automatically to the data using the R function dredge from the R package MUiMin (R version 3.0.2). Data were weighed according to the square root of the number of replicates in the study concerned.

Table 1 *Description of explanatory variables and factors used for model fit. Column 'added' indicates the order in which the explanatory variable were included in model when generating the most complex linear mixed model.*

Factor	Added	Classes	Description
temperature	1	continuous scale	14 to 35°C
origin	2	Asian; invasive	area of origin of ladybirds
photoperiod	3	short; long; natural	photoperiod during experiment: light during 14–15.5 hours, 16–18 hours, or naturally occurring (including unknown photoperiod)
food	4	Aphid; *Ephestia*	food of ladybird[c] used during experiment: aphids only or *Ephestia kuehniella* eggs (with or without aphids)
strain	5	field; reared	source of population: field-collected population (incl. subsequent short-term rearing of max. 4 generations) or laboratory reared population
sex[a]	6	female; male	
origin-photoperiod[b]	A[d]	Asian&short; Asian&long; invasive&long	origin and photoperiod combined, because factors were confounded

[a] *for response variable longevity*
[b] *for response variables survival L1, L2, L3, L4, and pupa*
[c] *of mother in case of eggs, of larva in case of pupa*
[d] *added after temperature and before food if origin and photoperiod were confounded*

The best models were selected with Akaike's Information Criterion (AIC), which is based on maximum likelihood. Because of small sample sizes, we used the corrected AIC (AICc).

$$\mathrm{AICc} = 2k - 2\mathrm{Log}(L) + \frac{2k(k+1)}{n-k-1},$$

with *Log* denoting the natural logarithm, *L* the maximum likelihood, *k* the number of estimated parameters, and *n* denoting the sample size.

The lowest AICc value indicates the best-fitting model. Models that differ less than 2 in AICc value are considered equivalent (Bolker 2008). Within the group of models with ΔAICc < 2, Ockham's razor (principle of parsimony) was used to choose the best model. Model selection with AICc is not based on the significance of the individual factors in the model, but it selects the overall best model. As a consequence the models are presented with the AICc value for the full model, and their relative believe (= AIC weight) within the models that differ less than 2 AICc points (Burnham and Anderson 2002).

$$\textit{AICc weight of model } i = \frac{\exp\left(\frac{-\Delta AICc_i}{2}\right)}{\sum_{i=1}^{m}\exp\left(\frac{-\Delta AICc_i}{2}\right)} \quad \textit{with}$$

$$\Delta AICc_i = AICc_i - \min_i AICc_i$$

Linear mixed models with and without temperature were used to model longevity (A4) and rates of development (D0 to D8), pre-oviposition period (A1), and generation time (A2). Fecundity (A3) models included temperature effect in polynomials of order 2 (quadratic), and survival models included temperature effect in polynomials of order 4 (quartic). A detailed description of methods is provided in appendix 1.

Data were lacking for Asian populations that had been *Ephestia* fed, that had been laboratory reared, or that had been both *Ephestia* fed and laboratory reared. For invasive populations, no data had been collected under short-day and natural-day conditions. Thus, the effects of photoperiod could only be estimated for Asian populations, while observed effects of food and ladybird strain are based on invasive populations ("Electronic Supplementary Materials A" (ESMA)). For several survival response variables, information on natural-day (except S0) and food (S1 to S5) was missing for both origins, while information on origin and photoperiod was confounded (S1 to S5). In a few instances, few studies were available, e.g. for most response variables the data for the aphid-fed, Asian population under short-day conditions are based on only two studies (Wang et al. 2009 (wild strain); Ware et al. 2009 (reared strain)).

To enable a comparison of Asian with invasive populations of *Harmonia axyridis* and to describe a "generic" invasive and a "generic" native *H. axyridis*, a selection of life history parameters is estimated. These parameters are estimated for wild, aphid-fed, Asian and invasive populations under long-day conditions, based on the best-fitting models, as far as the models differentiated for those subgroups (table 2) at four temperatures in the data range.

The second main question of this review is whether we can find potential explanations for the invasion success of *H. axyridis* when we compare data for wild, invasive, aphid-fed populations of *H. axyridis* with the life history data of the generalist native species *Adalia bipunctata* (L.), *Coccinella septempunctata* L., and *Propylea quatuordecimpunctata* (L.). To be able to do this, we first collected temperature data during the period that the ladybird beetles are active in the Netherlands in order to determine the temperature ranges for which European life history data need to be compared. Next, data on total immature development, longevity, and fecundity were collected for the three native species and weighted averages were calculated for the temperature ranges 14–16°C, 17–20°C, and 21–24°C as long as data were available. If available, data of wild, aphid-fed populations were used. Finally, data of several life cycle parameters such as development threshold, thermal constant, and voltinism were collected for both *H. axyridis* and the native species.

Results

Temperature had a large and consistent influence on life history parameters. The other factors are included in less than half of the twenty-two best models in total: origin nine times, photoperiod eight times, food four times, and strain five times. Data from eighty studies were used (figure 2 and supporting materials ESMA). For the best-fitting model of each response variable a graph, with parameters and formula, is given in ESMB. The twenty best-fitting models with AICc and factors that are included can be found in ESMC.

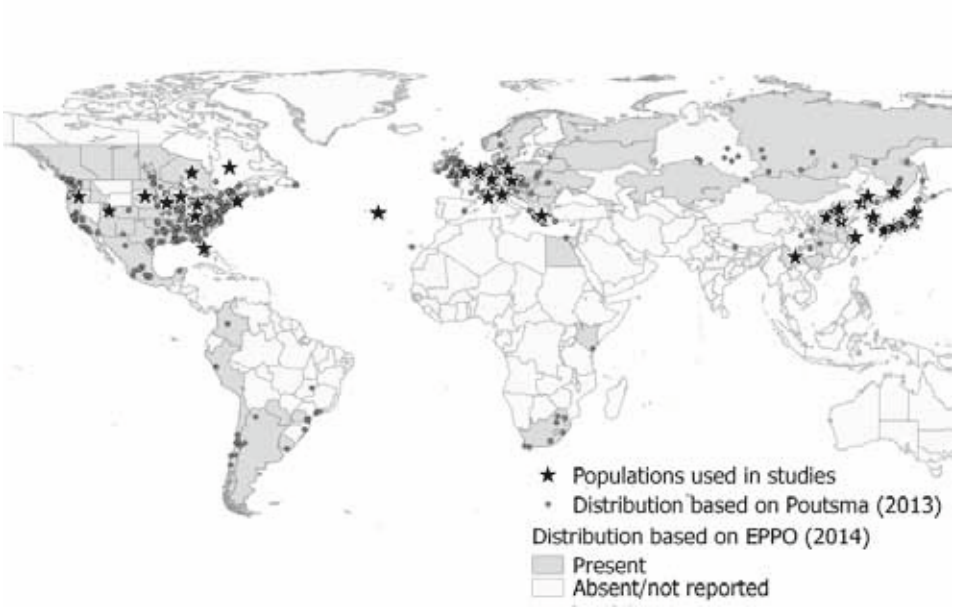


Figure 2 *Locations of* Harmonia axyridis *populations. Black squares show locations of populations used in the 80 studies analysed in this paper, grey dots show locations where* Harmonia axyridis *has been reported (based on Poutsma 2013).* Harmonia axyridis *is present in shaded countries, according to EPPO (2014).*

Models for development rate

All models for development (D0—D8) show a linear increase of development rate with temperature.

Immature stages

Immature development rate (D8, figure 3d) is higher at higher temperatures. Invasive populations have a stronger response to temperature, with beetles developing faster at high temperatures than those in Asian populations at the same photoperiod. Both in Asian and invasive populations, development is faster under short-day than under long-day conditions. In Asian populations development is fastest under natural-day length.

Egg

The best model for egg development rate (D0, figure 3a) only contains strain as a factor modulating the temperature effect. Eggs from wild strains develop faster than reared eggs at all temperatures. In the second best model for egg development (ΔAICc = 0.09) origin is included: eggs from Asian populations developed faster than eggs from invasive populations.

Larva

The best model for larval development (D5, figure 3b) contains origin as explanatory effect, in interaction with temperature. The response to temperature is stronger for invasive populations than for Asian populations. Thus, invasive populations develop faster than Asian populations at high temperatures. This effect of origin is in line with the effect observed in the model for total immature development (D8) under short-day. Models for the individual larval stages of second, third, and fourth instar (D2 to D4, for graphs see appendix 2) show similar patterns as the entire larval stage (D5). In contrast, the model for the first larval instar (D1, for graph see appendix 2) has photoperiod as explanatory factor in addition to temperature. At high temperatures development is faster under long-day. This effect is in contrast with the model for immature development (D8), where development is always faster under short-day.

Pupa

The best model for pupal development (D6, figure 3c) only contains a response to temperature. Other factors did not result in an improvement in fit that justified the extra parameter(s).

Larva+pupa

The best larva+pupa model (D7, for graph see appendix 2) contained only photoperiod as an explanatory variable interacting with the effect of temperature. The response to temperature is strongest for populations under long-day, weaker under natural-day, and weakest under short-day. At low and intermediate temperatures differences are small, but at high temperatures populations developed fastest under long-day, which is not consistent with the model for immature development (D8).

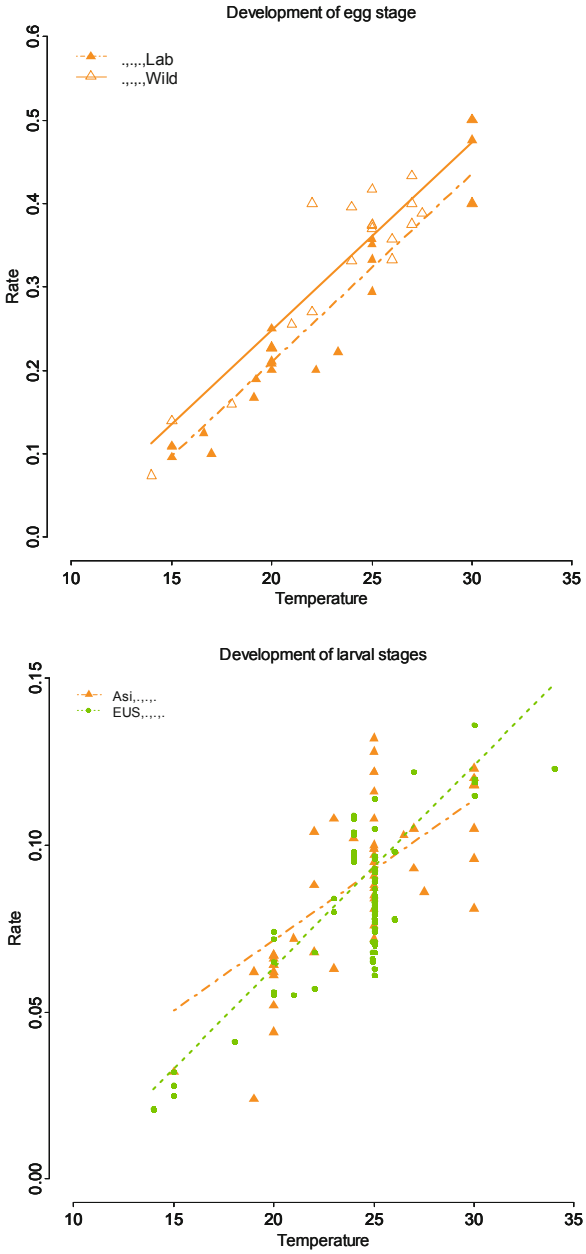


Figure 3 *Development rate of egg (a: D0), larva (b: D5), pupa (c: D6), and immature stages (d: D8) of* Harmonia axyridis. *Data points from literature are represented by symbols. Fitted relationships of the best models, i.e. the most parsimonious model within Δ AICc < 2, are represented by lines that are only shown when data are available for that set of factors.*

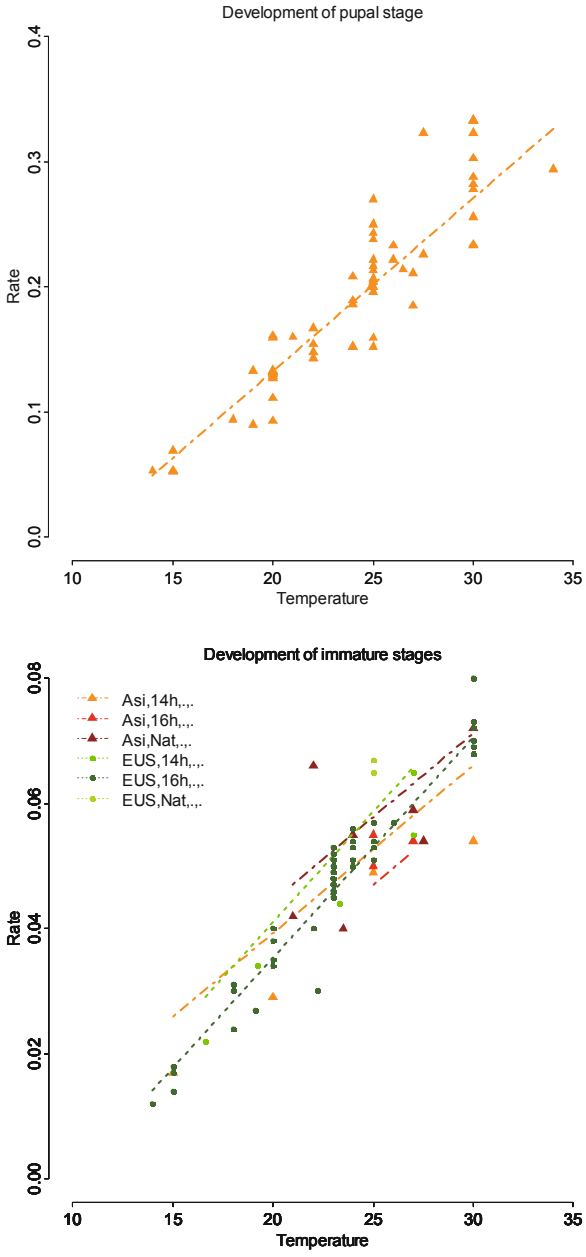


Figure 3 continued *Legends show the combination of factors. Geographic origin: Asia or invasive (EUS). Photoperiod: short (14h), long (16h), or natural (Nat). Strain: wild (Wild) or reared (Lab). When no distinction is made between the classes of a factor, that factor is represented by '.'. The x-axis shows temperature starting at 10°C.*

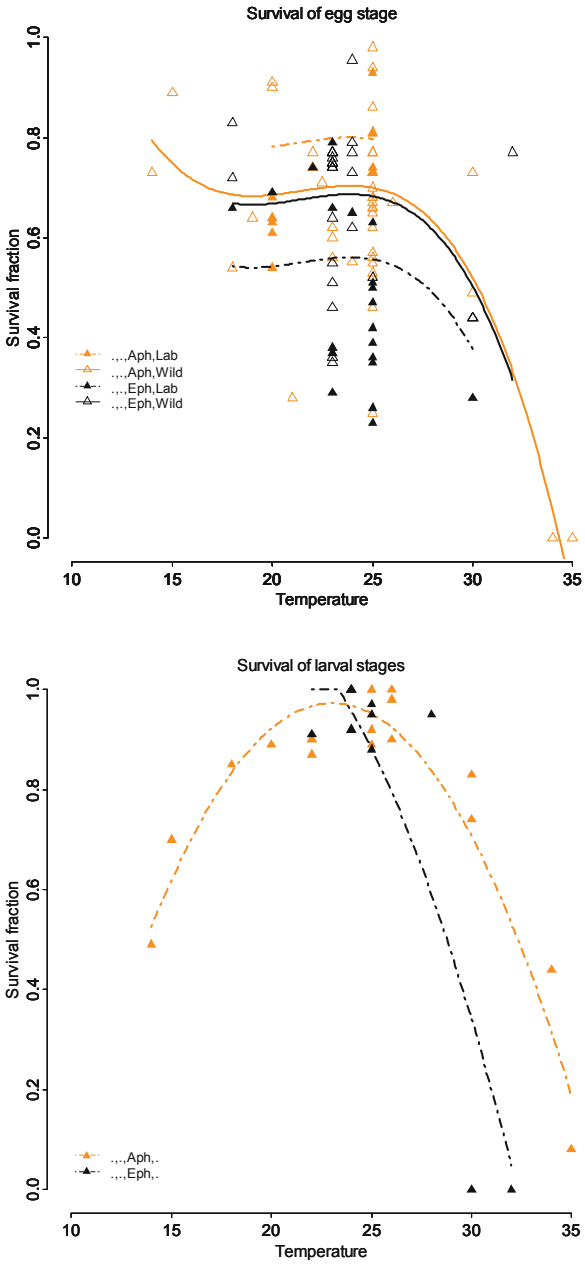


Figure 4 *Survival of egg (a: S0), larva (b: S5), pupa (c: S6), and immature stages (d: S8) of* Harmonia axyridis*. Data points from literature are represented by symbols. Fitted relationships of the best models, i.e. the most parsimonious model within Δ AICc < 2, are represented by lines that are only shown when data are available for that set of factors.*

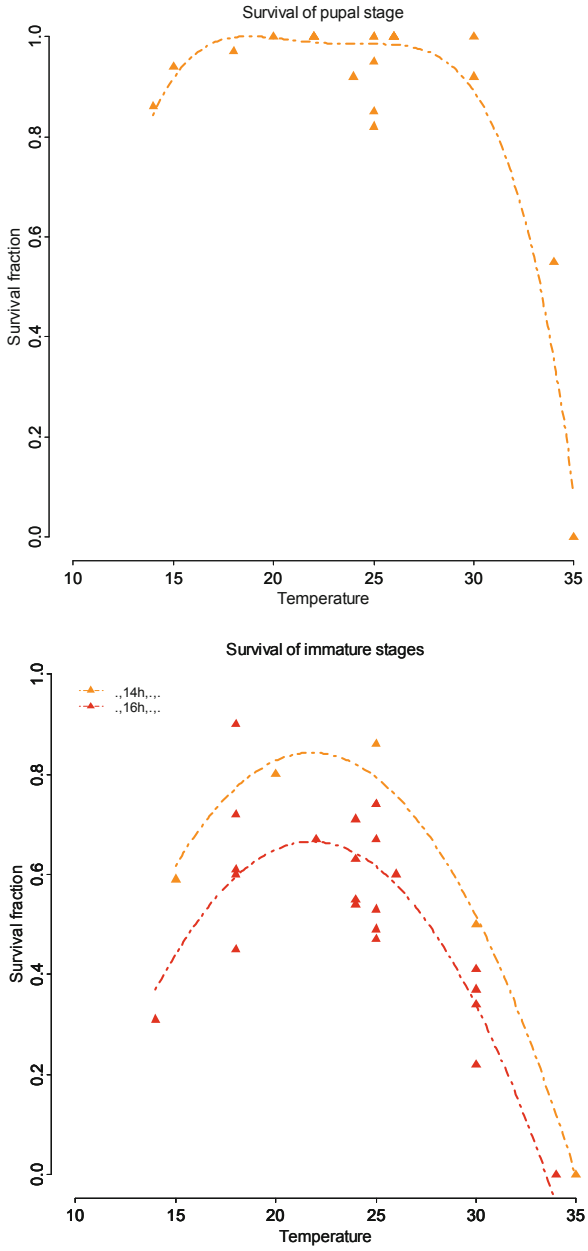


Figure 4 continued *Legends show the combination of factors. Photoperiod: short (14h), long (16h), or natural (Nat). Food: aphid (Aph) or* Ephestia *(Eph). Strain: wild (Wild) or reared (Lab). When no distinction is made between the classes of a factor, that factor is represented by '.'. The x-axis shows temperature starting at 10°C.*

Models for survival

All models for survival (S1 to S8), except survival of the eggs, have an optimum curve for temperature, with the optimum in the range of 20°C to 25°C. Survival of most stages decreases to zero at temperatures around 35°C. Temperature has a strong effect in the models for survival and is best described with a polynomial of order between two and four. If an additional explanatory variable is included in the best model, the pattern is not consistent: geographic origin is included in two best models (S3-combined with photoperiod, S7); photoperiod in three (S3-combined with geographic origin, S7, S8), food in two (S0, S6), and strain in only one best model (S0).

Immature stages

Maximum immature survival (S8, figure 4d) is 67% or 84% at approximately 22°C. In the best model temperature was included as covariate and photoperiod was included as an additive effect. Populations under short-day conditions have a higher survival.

Egg

The optimum temperature for egg survival (S8, figure 4a) is approximately 24°C. Egg survival depends on the food of the adults and whether the adults were laboratory reared or collected from the wild. Eggs from aphid-fed populations survive better than those from *Ephestia*-fed populations. Survival of wild populations is intermediate, while reared, aphid-fed populations survive best (80% at 24°C) and reared, *Ephestia*-fed populations worst (56% at 24°C).

Larva, pupa

Maximum survival of the individual larval stages (S1 to S4) and the pupal stage (S6, figure 4c) is more than 90%. The models for the individual stages of first, second, and fourth instar, and pupa (S1, S2, S4, S6, for graphs see appendix 2) all contain only temperature as explanatory variable. The larva model (S5, figure 4b) contains food, apart from the cubic relation with temperature, as additional explanatory variable. However, the absence of data at lower temperatures and the high leverage points at 30°C and 32°C result in a relationship that does not match survival relationships in general.

Larva+pupa

The best model for larva+pupa (S7, for graph see appendix 2) contains the factors origin and photoperiod and their interaction. Under long-day conditions invasive populations survive better than Asian populations, while under short-day conditions the effect is opposite.

Models for adult stage

Post-eclosion development

Pre-oviposition period

In the model for the inverse pre-oviposition period (A1, figure 5a) all explanatory variables, except origin, are included. Aphid-fed populations (both wild and reared) under long-day conditions show the strongest response to temperature, developing fastest at high temperatures (above 22°C). At high temperatures the longest pre-oviposition periods are observed for *Ephestia*-fed populations, and for wild, aphid-fed populations under natural-day. Within aphid-fed populations the temperature response under long-day is stronger than under short-day, as was observed for larval+pupal development (D7). Wild populations respond more strongly to temperature than reared populations.

Generation time

The inverse of generation time (A2, figure 5b) increases with increasing temperature, resulting in shorter generation times at higher temperatures. The response to temperature is stronger under long-day than under short or natural-day; this is in line with pre-oviposition period (A1) but in contrast to immature development (D8), which is always fastest under short-day. Reared populations have shorter generation times than wild populations at all temperatures.

Longevity

Most of the non-hibernating adults of *H. axyridis* have longevities between 26 and 100 days, with eight outliers between 117 and 170 days. Longevity (A4, figure 5d) decreases with temperature. Average longevity is longest for wild, Asian populations (88 days at 20°C) and shorter for reared, invasive populations (76 days at 20°C). For wild, invasive populations data at 23°C and 24°C are available. This subgroup significantly differs from the other subgroups and at those temperatures the model predicts longevity of 113 days: the longest of all populations. However, the small temperature range hinders a robust comparison. The best model does not contain an effect of sex, photoperiod, food, or strain.

Reproduction

The model for average daily fecundity (A3, figure 5c) is an optimum curve for temperature. Fecundity is highest for *Ephestia*-fed, invasive populations (31.0 eggs/day at approximately 27°C) and lowest for aphid-fed, Asian populations (25.8 eggs/day at approximately 26°C). Remarkably, aphid-fed, invasive populations have a much lower optimum temperature (below 20°C) for maximum fecundity (27.3 eggs/day) than the other two populations. All data points at 20°C, except one, originate from two studies performed on the Azores, where earlier generations were given a mixture of two aphid species and *Ephestia*, while the tested beetles were given only aphids (Soares et al. 2001; 2004). In addition, no data points at more extreme temperatures are available, and the low values at 25°C caused high leverage. We therefore suggest that the fitted relationship might be an artefact. More data at different temperatures are needed to obtain a better insight in the optimum temperature for fecundity of aphid-fed

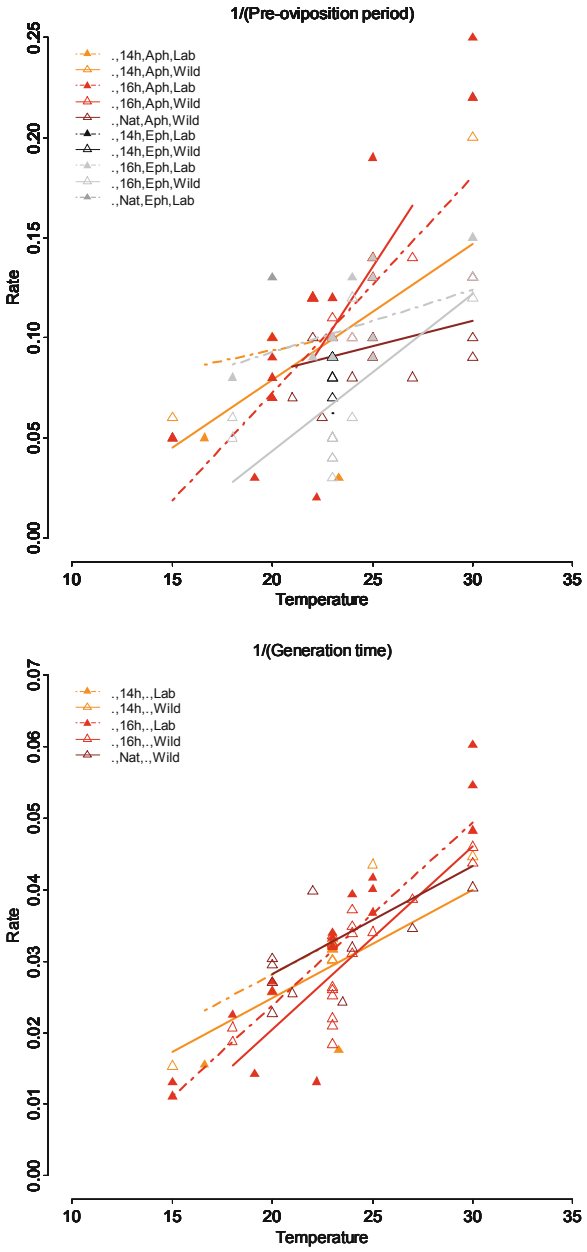


Figure 5 *Adult parameters 1/pre-oviposition period (a: A1), 1/generation time (b: A2), daily fecundity (c: A3), and longevity (d: A4) of* Harmonia axyridis*. Data points from literature are represented by symbols. Fitted relationships of the best models, i.e. the most parsimonious model within Δ AICc < 2, are represented by lines that are only shown when data are available for*

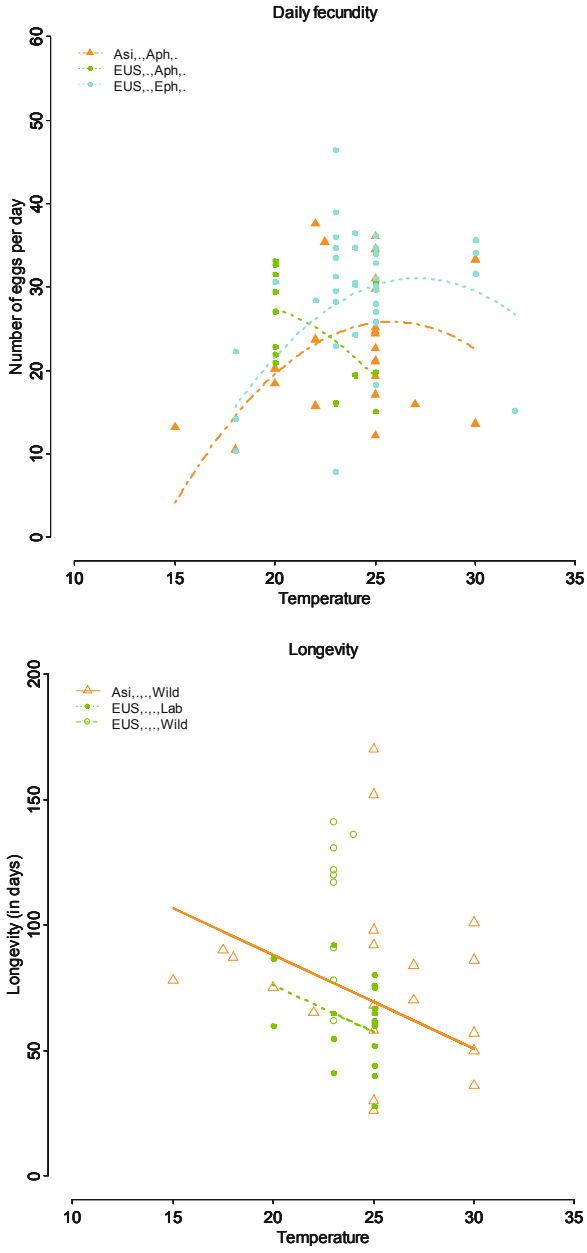


Figure 5 continued *that set of factors. Legends show the combination of factors. Geographic origin: Asia or invasive (EUS). Photoperiod: short (14h), long (16h), or natural (Nat). Food: aphid (Aph) or* Ephestia *(Eph). Strain: wild (Wild) or reared (Lab). When no distinction is made between the classes of a factor, that factor is represented by '.'. The x-axis shows temperature starting at 10°C.*

ladybirds. Daily fecundity measured over a period of less than fifteen days (7–39 eggs/day) is similar to daily fecundity measured over a period of more than forty days (10–37 eggs/day). We therefore did not distinguish between observation periods of different lengths.

Data outside defined range: extreme photoperiods

For several response variables an effect of photoperiod was found. Data from several studies show that *H. axyridis* is also able to survive, develop, and reproduce under extreme photoperiods like continuous light or continuous darkness (three and nine studies, respectively). In general, weighted average values for aphid-fed ladybirds under extreme photoperiod are within the same range as the data used for our model fit (appendix 3), except those for egg and immature development (D0, D8), larva+pupa and immature survival (S7, S8), and longevity (A4). In contrast, values for ladybirds fed an artificial diet are generally lower than data used for fitting the models.

Estimated life history data for wild, aphid-fed populations under long-day conditions

To enable a comparison of Asian with invasive populations of *Harmonia axyridis* and to describe a "generic" invasive and a "generic" native *H. axyridis*, a selection of life history parameters for wild, aphid-fed Asian and invasive populations under long-day conditions is estimated as far as the models differentiated for those subgroups (table 2) at four temperatures in the data range. Compared with Asian populations, larval development time of invasive populations is shorter above 25°C, immature development time is shorter at 20°C and above, and fecundity is higher at 20°C and above. Longevity of invasive populations is longer at all temperatures. For egg and pupal development time, immature survival, and pre-oviposition period no distinction can be made between the two origins. The developmental threshold of invasive populations is higher, but the thermal constant is lower than that of Asian populations.

Life cycle parameters of native species

Figure 6 shows that maximum temperatures in the Netherlands in summer (early August) do not exceed 23.5°C, while average temperature in the same period is 18.3°C. In order to address the second main question of this review, the comparison with native species, a summary of the life cycle parameters of the four species *A. bipunctata, C. septempunctata, P. quatuordecimpunctata,* and *H. axyridis* is given in table 3. A more extensive overview, including references, can be found in the supplementary materials (ESMD and appendix 4). Table 3 shows that *H. axyridis* and *C. septempunctata* have the highest threshold temperature and thermal constant, and both species develop relatively slowly, while *P. quatuordecimpunctata* is faster and *A. bipunctata* develops fastest. The body size of *H. axyridis* and *C. septempunctata* is similar and larger than that of *A. bipunctata* and *P. quatuordecimpunctata*. Highest fecundity is reported for *H. axyridis*. A comparison of voltinism of the species shows that *H. axyridis* is the only species that has two or more generations per year, while the other species at most only have a partial second generation in northwestern Europe.

Table 2 *Estimated values of life history parameters for Asian and invasive populations (invas) of* Harmonia axyridis

Temp	Development time (days)						Survival	Pre-ovip.[ac] (days)	Daily fecundity		Longevity (days)		Threshold (°C)		Thermal constant (°C-days)	
	egg[a]	larva		pupa[a]	immature		immature[a]									
	both	Asian	invas	both	Asian[b]	invas	both	both	Asian	invas[e]	Asian	invas[d]	Asian	invas	Asian	invas
15	7.4	19.8	30.5	15.8	*49.8*	56.6	44.0	*Na*	4.1	3.7	107	*143*	7.48	10.03	372.8 – 374.4	281.5 – 283.6
20	4.0	14.0	15.9	7.5	*29.8*	28.4	65	*17.2*	19.6	21.6	88	*124*				
25	2.8	10.7	10.8	5.0	*21.3*	18.9	61.8	*7.4*	25.7	30.2	69	*105*				
30	2.1	8.1	8.8	3.7	*16.6*	14.2	33.9	*4.7*	22.6	29.5	51	*87*				

Estimates are given for wild, aphid-fed populations under long-day conditions, as long as the best model differentiates for these factors (ESMC). Developmental threshold and thermal constant are given for immature development (S8). The best models for egg development[a] (D0), pupal development[a] (D6), immature survival[a] (S8), and pre-oviposition period[a] (A1) do not contain an origin effect, therefore values for Asian and invasive populations are identical. Estimates in italics are based on only three[b] data points, or on a small temperature range (22–27°C)[c], or on nine data points at one temperature (23–24°C)[d], and might not be valid outside the data range. For fecundity invasive aphid-fed populations had an unrealistically low optimum temperature, therefore values of invasive[e] Ephestia*-fed populations were used.*

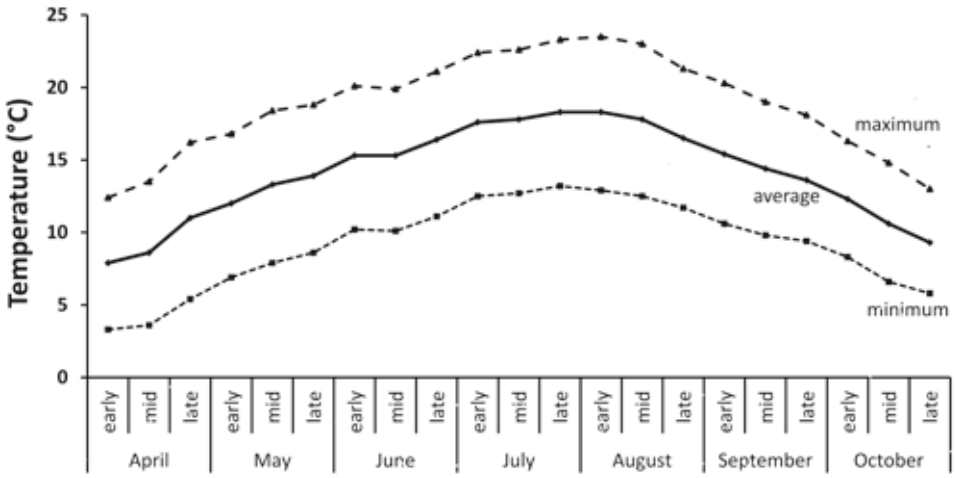


Figure 6 *Long-term (30-year) averages for minimum, maximum, and daily average temperatures in spring, summer, and early autumn in the Netherlands (weather station De Bilt, KNMI 2013)*

Table 3 *Characteristics for wild, European, aphid-fed populations of four generalist coccinellid species:* Harmonia axyridis *(Hax),* Adalia bipunctata *(A2),* Coccinella septempunctata *(C7), and* Propylea quatuordecimpunctata *(P14). Individual values of given ranges and weighted averages and their references are given in ESMD. Developmental threshold and thermal constant are given for immature development (D8).*

Species	Immature development (days)			Longevity (days)		Fecundity daily		Fecundity total (N)		Threshold (°C)			Thermal constant (°C-days)			Voltinism in NL (N/year)	Spatial-temporal co-occurrence *H. axyridis*[k]	Size[l] (mm)	Intraguild predation (rel. to *H. axyridis*)	Population trend since arrival *H. axyridis*[n]	
	14–16°C	17–20°C	21–24°C	17–20°C	21–24°C	17–20°C	21–24°C	17–20°C	21–24°C	Europe	N-Am	Asia	Europe	N-Am	Asia					distribution	abundance
Hax[a]	56.6	35.4	23.6	131.4[b]	116.5[b]	15.5[c]	26.1[c]	1468[e]	2430[e]	10.5-11.2; 10.09[f]	11.2	7.48[f]	231.3-258.3; 281.8[f]	267	330.4[g]; 373.6[f]	2, partially 3		4,9–8,2	Predator		↑ BE, CH, UK
A2	44.3[d]	19.7	15.8	74.9[d]	73.3	11.5[d]	20	968[d]	1011	8.5-10.06	9	6.3	244.8-267.9	262.8	322.6	1, partially 2	61.2%	3,5–5,5	Prey	↓30% BE, ↓44% UK	↓ BE, CH, UK
C7	62.3	37.9	25	-	57.3	24.9[h]	27.7	1177[ei]	1310[e]	10.5-12.4	12.1	11.1	206-281.5	196.8	209.9[g]	1, rarely 2	53.3%	5–8	Prey	→ BE, UK	→ UK (no data BE, CH)
P14	53.5	23.9	20.4[d]	120.4	67.9	1.9	6.1[j]	465	359	9.9-15	9.9	na	144-218	207	na	1, partially 2	52.2%	3,5–5,2	Prey	↓ BE, UK	↓ CH (↓ns BE, UK)

[a] *Values for* Harmonia axyridis *for development, longevity and fecundity are based on fitted relationships. Reported values are based on the differentiation in factors made in the best model of each response variable (see ESMC)*

[b] *Based on extrapolation of data at 23–24°C*

[c] *Based on* Ephestia*-fed population*

[d] *Based on reared populations*

[e] *Estimated by: daily fecundity*(longevity–pre-oviposition period)*

[f] *Based on best model of fitted relationship*

[g] *Sum of separate values for egg, larva and pupa*

[h] *Overwintering population*

[i] *For longevity and pre-oviposition data at 21–24°C were used*

[j] *Estimated by: total fecundity/(longevity–pre-oviposition period)*

Data from: [k] *Adriaens et al. (2008);* [l] *Iablokoff-Khnzorian (1982);* [m] *Pell et al. (2008);* [m] *Chapter 6;* [n] *Roy et al. (2012)*

Discussion

Factors influencing life histories of native and invasive *Harmonia axyridis*

Origin

The first aim of this review was to determine whether invasive populations differed in life history characteristics from native Asian populations. A difference between invasive and Asian populations was found for nine of the twenty-two response variables, especially in the models that describe development (D2 to D4, D6, D8, D7-third best model); those show invasive populations responding stronger to temperature, developing faster than Asian populations at high temperatures. Invasive adults also have a higher daily fecundity (A3). In contrast, Asian populations have a faster egg development (D0, second model) than invasive populations. So invasive populations do indeed differ from Asian populations.

Variation in life history traits between coccinellid populations from different geographic origins has been found in some studies (Obrycki and Tauber 1982; Michels and Flanders 1992) but not in others (Obrycki and Tauber 1981; Miller 1992; Obrycki et al. 1993; Phoofolo and Obrycki 1995). Rapid evolutionary changes during invasion are common in plant and animal species (Whitney and Gabler 2008). The difference between invasive and Asian populations of *H. axyridis* observed in our meta-analysis could possibly be explained by the following mechanisms: 1) sampling effect: a non-random sample with a strong temperature response is the source of the invasive populations; 2) admixture of two geographically distinct Asian populations (Blekhman et al. 2010; Brown et al. 2011b; Lombaert et al. 2011), which in turn have admixed with the European biocontrol strain (Lombaert et al. 2010); 3) purging of deleterious alleles at the bottleneck of the invasive process (Facon et al. 2011b); 4) captivity rearing, stimulating fast development and high fecundity, which has changed the biocontrol strains that have been introduced (Tayeh et al. 2012); and 5) unbalanced set of available data as half of the data of the invasive population originate from reared populations (47%), compared with only 10% of the data of the Asian populations (ESMA).

In this meta-analysis we found higher fecundity for invasive populations, but we did not find a shorter pre-oviposition period as suggested by Laugier et al. (2013). Therefore, our analysis only partly supports the hypothesis that a higher reproductive investment (i.e. higher fecundity and shorter pre-oviposition period) is an advantage in the early stages of invasion, when population densities are low, because it speeds up population growth (Laugier et al. 2013, not included in the dataset).

Although invasive populations develop faster than Asian populations at high temperatures, there is no difference between Asian and invasive populations at intermediate temperatures that are, for example, representative of western European spring and summer (17–24°C). Hence, this meta-analysis does not show that differences in these life history characteristics have promoted the invasion of *H. axyridis.* Although it has been postulated that hybridisation and purging of deleterious alleles may contribute to the success of invasive species (Facon et al. 2011b), Tayeh et al. (2013) did not observe any differences in generation time and 'life time performance' between wild, invasive and wild, Asian populations and their hybrids. We agree with their conclusion that "genetic admixture *per se* is unlikely to have

significantly contributed to the invasion success of *H. axyridis*". Nevertheless, a strong inbreeding depression was observed for wild Asian populations and not for wild invasive populations when crossing siblings, demonstrating that deleterious alleles were purged in the invasive population (Facon et al. 2011b, not included in analysis because of foodtype). This inbreeding depression observed under laboratory conditions in Asian populations is likely to be counteracted by a large effective population size in the field in Asia and matings between siblings are probably rare in the wild (Tayeh et al. 2013).

Field versus controlled conditions

In our meta-analysis we can only focus on differences under controlled laboratory conditions between invasive and Asian populations. Field data are collected at fluctuating, unknown temperatures, and therefore cannot be used for model fitting. The disadvantage of this approach is that characteristics selected for by rearing under laboratory conditions are often deleterious under field conditions (Tayeh et al. 2012), and a wild population brought under controlled conditions may suffer adaptation stress. The performance in the field is determined by many factors and conditions.

The few available field data (ESME, select for field and semi-field conditions: type = 2 or 3) are in the range of the data used for the model fitting. Development data (D5–D8, Sakurai et al. 1993) are close to the relationship fitted for the corresponding populations, while data for longevity (A4, Katsoyannos et al. 1997a) are higher than the fitted relationship and those for fecundity (A3, Bazzocchi et al. 2004) are lower. In contrast, survival (S0–S8) observed in the field is often substantially lower than in the laboratory, and only values for egg survival are similar.

Our models suggest that most variation in immature survival (S8) is caused by the egg stage (S0), as the egg stage model also shows considerable variation, while the models of the individual stages do not (always more than 90% survival). However, in the field the larval stages explain most variation in survival, not the egg stage (Osawa 1993; Kindlmann et al. 2000). This discrepancy between field and laboratory data for survival tells us to be cautious when extrapolating laboratory or model data to field circumstances.

Temperature

The covariate temperature is included in all twenty-two best models. The observed relationships follow relationships as expected from literature (e.g. see Miller 1992; Omkar and Pervez 2004; Jalali et al. 2010; Nedved and Honek 2012): 1) a linear relationship with temperature within the temperature range of 14–35°C for longevity (A4) and for the rate of development (D0–D8), pre-oviposition period (A1), and generation time (A2); and 2) an optimum curve for temperature for survival (except egg survival) and fecundity. For the survival parameters, temperature was often even the only explanatory variable.

Photoperiod

The factor photoperiod is included as explanatory variable in the models of eight response variables, five of which cover development (D1, D7, and D8), pre-oviposition

period (A1), and generation time (A2). In these models short-day conditions flatten the response to temperature resulting in faster development under long-day conditions at higher temperatures. The exception is immature development (D8) where development is fastest under short-day. The effect of photoperiod on survival is not consistent. The only study that experimentally determined the effect of several photoperiods of fourteen hours and longer on development (D5–D7, A1), of reared, Asian, aphid-fed populations (20°C), did not find any effect (Reznik and Vaghina 2011) (ESME).

Food

Food emerged as an explanatory variable in only four out of twenty-two models, and none of them was a model for developmental rate. The estimated effects are not very consistent. Aphid-fed populations have a higher survival (S0, S5) and generally a shorter pre-oviposition period (A1) but a lower fecundity (A3) than *Ephestia*-fed populations. Almost no data are available for Asian *Ephestia*-fed populations, and half of the invasive *Ephestia*-fed populations are laboratory strains: food type effect is confounded with origin and strain. Several individual studies directly compared the effect of *Ephestia* and aphid diets, but the results were not consistent over those studies (Schanderl et al. 1988; Specty et al. 2003 (flightless beetles); Baldacci 1998 in Lanzoni et al. 2004; Berkvens et al. 2008b; Kögel et al. 2012a; Nedved and Kalushkov 2012; Rodrigues et al. 2013 (12 hours light)). This meta-analysis does not shed light on the inconsistency of the results. The absence of a food effect in most models supports the idea that the generalist *H. axyridis* is not so much affected by food quality but rather by other factors. Nedved and Kalushkov (2012) for example, showed that several characteristics of *H. axyridis* were differentially influenced by air humidity depending on the food type (aphid or *Ephestia*).

Ladybird strain

In general, laboratory rearing selects for fast development, short longevity, and high fecundity (Tayeh et al. 2012). Effect of rearing is not very consistent in the individual studies that compare reared and wild strains (Berkvens et al. 2008a; 2008b; Lombaert et al. 2008; Facon et al. 2011a (artificial diet); Turgeon et al. 2011). In our meta-analysis only five best models include the factor strain, three of which indeed show that egg development time (D0), generation time (A2), and longevity (A4) of reared strains is shorter while the pattern in the other two is less clear. However, "strain" is often confounded or has an interaction with origin and food, obscuring strain effect.

8

***Harmonia axyridis* and competition with native species in Europe**

In answer to the second main question of this review, 'do differences between native European ladybirds and *H. axyridis* provide an explanation for this invasiveness?', we compare and discuss the life cycle parameters of the invasive *H. axyridis* and the generalist native species *A. bipunctata, C. septempunctata*, and *P. quatuordecimpunctata,* below.

Harmonia axyridis and *C. septempunctata* develop relatively slowly compared with *P. quatuordecimpunctata* and *A. bipunctata* (table 3). The relatively high

threshold temperature in combination with the relatively high thermal constant also indicates that both *H. axyridis* and *C. septempunctata* develop slowly, in contrast to *A. bipunctata*, which has a low threshold temperature, and *P. quatuordecimpunctata*, which has a low thermal constant (Obrycki and Tauber 1981; Hemptinne et al. 1988). As survival under laboratory conditions differs greatly from survival under field conditions, we did not make a comparison for that life history characteristic. The few field studies that compare survival of a native species and *H. axyridis,* show a higher survival for *H. axyridis* compared to *A. bipunctata* (chapter 5) and to *C. septempunctata brucki* (Osawa 1993; Yasuda and Shinya 1997; Kindlmann et al. 2000).

Occurrence of native species is synchronised with peaks in aphid abundance in wheat (*A. bipunctata* (Hemptinne et al. 1992), *C. septempunctata* (Honek 1980; Basedow 1982), and *P. quatuordecimpunctata* (Hemptinne et al. 1988)), in potato (*P. quatuordecimpunctata* (Jansen and Hautier 2008) and *C. septempunctata* (Jansen and Hautier 2008)), in alfalfa (*C. septempunctata brucki* (Takahashi 1989)), in hibiscus (*C. septempunctata brucki* (Yasuda and Shinya 1997)), and in lime trees (*A. bipunctata* (Wratten 1973)). *Harmonia axyridis* populations are not synchronised with peaks in aphid abundance. They arrive and reproduce relatively late when compared with aphid densities and native species (Ameixa et al. 2013) in potato (Jansen and Hautier 2008), broad bean and maize (Vandereycken et al. 2013a), alfalfa (Takahashi 1989), and hibiscus (Yasuda and Shinya 1997; Kindlmann et al. 2000). A consequence of the late start of reproduction in combination with the relatively slow development is that late instar larvae of *H. axyridis* have to complete development when aphid densities are low (Jansen and Hautier 2008); they effectively use other ladybird species as food source (Ameixa et al. 2013).

Harmonia axyridis is a strong intraguild predator (e.g. Pell et al. 2008; Ware and Majerus 2008) that is known to be able to survive and complete development on conspecific and heterospecific eggs, larvae, and pupae (Yasuda and Ohnuma 1999; Cottrell 2004; Sato and Dixon 2004; Sato et al. 2008; Ware et al. 2009). Sato et al. (2003) even showed that under conditions of prey extinction *H. axyridis* was not able to reach the pupal stage when only conspecific larvae were available as food, while it was able to survive when placed together with heterospecific larvae. For intraguild predation to occur under natural conditions, habitat overlap of immature stages is needed. Adriaens et al. (2008) (table 3) report a habitat overlap of 50% or more between *H. axyridis* and native species, but they do not discriminate between immature stages and adults. In the UK greater niche overlap between *H. axyridis* and native species was associated with a stronger population decline of the native species (Comont et al. 2013).

Compared to the three native species *H. axyridis* seems to use a wider range of habitats. *Adalia bipunctata* is said to be a species of shrubs, trees (Nedved 1999), and herbaceous weeds (Honek and Rejmanek 1982; Majerus 1994), while *C. septempunctata* and *P. quatuordecimpunctata* are common in field crops (Honek and Rejmanek 1982; Hemptinne and Naisse 1988; Nedved 1999). However, all three have been observed in other habitats as well: *C. septempunctata* and *P. quatuordecimpunctata* on trees and wild herbs (Honek 2012) and *A. bipunctata* on various crops (Hemptinne and Naisse 1988; Honek 2012). *Harmonia axyridis* favours tree and shrub lands, both in

native and invaded areas, but it also occurs in herbaceous and urban habitats such as parks and gardens (Adriaens et al. 2008; Osawa 2011; Vandereycken et al. 2012). In Japan and North America *H. axyridis* also occurs in orchards (Osawa 2011; Honek 2012). In the agricultural landscape *H. axyridis* has become dominant in many field crops in North America (Alyokhin and Sewell 2004; Hesler and Kieckhefer 2008 ; Schmidt et al. 2008; Honek 2012). In Europe this dominance has not been observed yet (Honek 2012). In corn and broad bean *H. axyridis* occurs and reproduces in higher densities than in potato and wheat. While *H. axyridis* has become the most abundant coccinellid in corn, *C. septempunctata* is most abundant in the other three crops (Vandereycken et al. 2013a; 2013b). *Harmonia axyridis*, *A. bipunctata*, and *C. septempunctata* co-occur on stinging nettles in field edges (Alhmedi et al. 2007; 2009).

The above data show that the three native species have a considerable overlap in habitat with *H. axyridis*. The next important question is whether they meet each other under field conditions. We found that the contact frequency in the field may not be as high as in laboratory experiments, but when the species meet, the outcome of a contact is similar as in the laboratory: *H. axyridis* wins the interaction (chapter 6). Three studies quantified intraguild predation in the field based on gut analysis. In *H. axyridis* larvae collected in lime trees *Adalia* spp., *Calvia* spp., and *P. quatuordecimpunctata* were found in Belgium (Hautier et al. 2011), and *A. bipunctata* and *A. decempunctata* (L.) were found in the UK (Thomas et al. 2013). In soybean fields in Canada *C. septempunctata, P. quatuordecimpunctata,* and *Coleomegilla maculata lengi* Timberlake were found in larvae of *H. axyridis* (Gagnon et al. 2011).

Since the arrival of *H. axyridis* in Belgium, UK, and Switzerland, *A. bipunctata* populations have declined. *Propylea quatuordecimpunctata* populations declined as well although not always significantly. *Coccinella septempunctata* populations appear to be stable (Roy et al. 2012, table 3). The fact that *C. septempunctata* seems to be affected less can be attributed to its large size and its habitat preference that differs from *H. axyridis* and, therefore, has the least overlap with *H. axyridis*. The difference between the two small ladybird species *A. bipunctata* and *P. quatuordecimpunctata* may be that the former has a higher degree of habitat overlap, especially on trees and shrubs early in the season (Brakefield 1984). Moreover, *P. quatuordecimpunctata* prefers the lower parts of plants compared to *A. bipunctata* (wheat, Hemptinne et al. 1988), *C. septempunctata* (Honek 2012), and *H. axyridis* (maize, Hoogendoorn and Heimpel 2004), probably reducing the chance of contact with *H. axyridis*. Indeed, the intraguild predation field studies show lower incidence of intraguild predation on *P. quatuordecimpunctata* than on other species (Gagnon et al. 2011; Hautier et al. 2011).

In this paper we have demonstrated that invasive populations of *H. axyridis* differ in some of their life history characteristics from the populations in its native area, which provides a starting point for further study into the possible mechanisms causing these differences. Such information will be crucial to predict future scenarios with respect to the invasion process of *H. axyridis*, especially in the light of ongoing climate change. The observed differences in life history characteristics primarily exist at temperatures above 25°C. Important traits associated with invasiveness are short generation time, high fecundity, and high survival resulting in a high growth rate (e.g. Whitney and Gabler 2008). When we consider those traits and the comparison with

native species made above, can we explain why *H. axyridis* is a successful invader in Europe? Although *H. axyridis* develops slowly and starts development late in the season in comparison with native species, we conclude that its high longevity and high fecundity, in combination with it being the strongest intraguild predator and having more generations per year, result in faster population growth than that of native species. This clearly contributes to the invasion success of *H. axyridis*.

Acknowledgements

We would like to thank the several people for contributions: all authors who provided raw data or further explanation on their data; D Lucas Barbosa, K Oku, C Ponzio, YT Qiu, S Wang, M Yajima, P Zhang, and S Zheng for help with translation of manuscripts; J Poutsma and PPL van Genuchten for constructing the *H. axyridis* distribution map; and SW Benedictus-van den Berg, C van Boxel, and MJ Raak for other support.

Electronic Supplementary Materials

Additional information may be found in the Electronic Supplementary Materials:
(http://edepot.wur.nl/306497)
ESMA: Overview data distribution in factors and classes (http://edepot.wur.nl/306541)
ESMB: Graph, parameters and formula of best model (http://edepot.wur.nl/306542)
ESMC: Best 20 models for each response variable (http://edepot.wur.nl/306543)
ESMD: Life history data native species plus references (http://edepot.wur.nl/306544)
ESME: Raw data plus references (http://edepot.wur.nl/306545)

Appendix 1 chapter 8

Methods

Data(base)

Data search

Studies that report experimental data on life history characteristics of *H. axyridis* were gathered from previously published reviews and by carrying out a literature search on the databases CAB abstracts, ISI web of Science, Biological records, Zoological records, and Agricola. Studies from 1900 onwards were surveyed. The last search was conducted in July 2013. Additional studies were found by searching in the bibliographies of papers. When abstracts of Chinese and Japanese studies were promising, relevant sections, tables, and figures were translated by native speakers. When the original paper could not be obtained or translated, the data that were given in a) the abstracts, b) figures and tables in English, or, c) review papers (only when the source was clear), were included in the database (for raw data and selected studies see Electronic Supplementary Materials E (ESME)).

Data that were presented in figures were digitalised using the Engauge Digitizer Version 4.1 and the resulting numbers were used. In other cases authors provided raw data. When ambiguity arose about definitions, details, or calculations used, clarification was asked from the authors (ESME). In case the author could not be reached and no funded assumption could be made, data were discarded.

Selection of studies

The studies had to meet some criteria to be included in the dataset. Only data that were determined under biologically relevant conditions were included. Therefore extreme photoperiods (< 14 or > 18 hours of light) and temperatures (< 14°C or > 35°C) were excluded. Field studies are conducted under fluctuating temperatures; therefore results of field studies could not be included in the analysis.

Although under natural conditions *H. axyridis* can experience situations with limited food and although the larvae of this generalist predator can prey and survive on non-aphid food, only data generated with unlimited access to aphids or *Ephestia kuehniella* Zeller (Lepidoptera, Pyralidae) (further referred to as *Ephestia*) as prey were included. This was done for three reasons: a) toxic or unsuitable aphid species (for definition see Hodek and Honek (1996)) and artificial diet often do not result in development at all; b) food limitation is difficult to quantify, and in general it directly affects development and survival; and c) results based on other non-aphid prey (e.g. ladybird eggs or larvae) are scarce and difficult to categorise and standardise. As many studies use *Ephestia* as food source, these studies were included. The majority of studies using artificial diet were conducted under extreme photoperiods and at 25°C. Studies that did not mention which prey was used, were left out as well. When food availability was not mentioned, we assumed food was unlimited. Aphid species (Hemiptera: Aphididae) excluded because of their unsuitability for development and/or reproduction were: *Aphis craccivora* Koch, *Aphis pomi* De Geer, *Aphis spiraecola* Patch, *Aulacorthum magnolia* (Essig & Kuwana), *Brevicoryne brassicae* (L.), *Hyalopterus pruni* (Geoffroy), *Megoura viciae* Buckton, and *Toxoptera citricidus* (Kirkaldy).

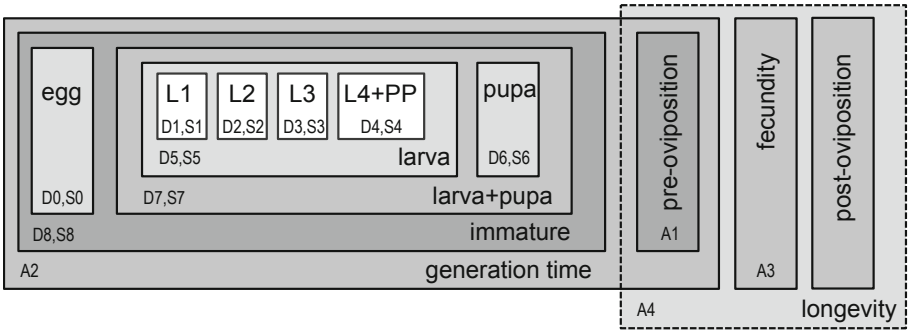
Studies determining life history characteristics (e.g. fecundity, survival, or longevity) of individuals that had hibernated or were hibernating were excluded. From studies with a manipulative treatment (e.g. insecticide treatment) only the data resulting from the control treatment were regarded as relevant and included in the database. Results by Thompson (1926; 1927) were excluded as well, as the species (*Leis* sp.) they worked with was not *H. axyridis*, but *Harmonia conformis* (Y. Jongema pers. comm., Laboratory for Entomology, Wageningen).

The survival of beetles of the flightless strain of *H. axyridis* was relatively low and had a different pattern than that of beetles that were able to fly. Therefore, all data for flightless ladybirds were left out of the dataset.

Response variables

Twenty-two life history characteristics were defined as response variable, being a measure for immature development, immature survival, post-eclosion development, and reproduction. In the database those studies were included that reported at least one of the life history characteristics or a variable that could be converted into a life history characteristic. SE, SD, range or other, given by author, were recorded as well.

The major difficulty was that different authors used different definitions, measurements, and methods for the life history characteristics. Therefore recalculations had to be made. When studies reported values for response variables that, after recalculation, did not fit the definitions of the response variables used in this paper, it was not possible to include the results in this meta-analysis.



Copy of figure 1 *A schematic overview of the life stages of* H. axyridis *used to define the life history characteristics and their relationships. L1, L2, L3, and L4 refer to four larval instars. The fourth instar includes the pre-pupal stage (PP). Codes at the bottom of each box refer to corresponding response variables and are referred to in the text (D0, D1, D2, D3, D4, D5, D6, D7, D8 code for development parameters; S0, S1, S2, S3, S4, S5, S6, S7, S8 code for survival parameters; and A1, A2, A3, A4 code for the parameters for post-eclosion (adult) development).*

Figure 1 of the main text (copy included here) gives a schematic overview of the life cycle of *H. axyridis* consisting of four larval instars and a pupal stage, separated by moults. The moult into the next stage is the cut-off for the earlier stage. The fourth instar has a mobile and an immobile (prepupal) phase, not clearly separated by a

moult. In this analysis the prepupal stage is considered as part of the L4 stage, therefore data are combined when authors report separate data for L4 and prepupa. When authors did not clearly define the stages, it was assumed that the authors included the prepupa in the fourth instar.

For both larval development rate and larval survival response variables were defined for the following nine developmental stages: egg, L1, L2, L3, L4, larva (from egg hatch to moult into pupa), pupa (from moult into pupa to adult emergence), larva+pupa (from egg hatch to adult emergence), and immature stages (from egg to adult emergence) (codes: D0 to D8 and S0 to S8, figure 1). When studies only gave values for individual instars, data were lumped together and combined to cover a longer period, only if those values were obtained from the same group of insects (e.g. separate development values for the instars L1+L2+L3+L4 are combined into one value for larval development). Although developmental time is often determined for larvae of unknown sex, while pre-oviposition period is determined for females, the values of immature development (D8) and pre-oviposition (A1) are combined to obtain more data for the response variable generation time (A2).

Development time is the length of time the larva needs to develop from one stage to the next. Development rate is the reciprocal of the development time. Survival is the fraction of larvae that develop into the next developmental stage. When mortality was reported it was recalculated into survival (= 1 – fraction mortality). The fraction of egg hatching is similar to egg survival.

After adult emergence the adult has a certain longevity (A4). This period can be split in a pre-oviposition period (A1) (from adult emergence to first oviposition), an oviposition period (A3) (from first to last oviposition), and a post-oviposition period (from last oviposition until death). Generation time (A2) is the time that elapses from egg deposition until the first oviposition by the resulting adult. Longevity (A4), the rate (reciprocal) of pre-oviposition period (A1), and the rate (reciprocal) of generation time (A2) are three response variables for the adult stage.

The fourth adult response variable is fecundity (A3), measured as the number of eggs laid. When fecundity was given as a total number over a certain period, it was recalculated into a daily fecundity (i.e. the average number of eggs oviposited per day over the studied period). When total fecundity was determined over the total period of longevity, which also includes the pre-oviposition period and the post-oviposition period (figure 1), dividing by longevity led to an underestimation of daily fecundity compared with daily fecundity that was measured over a short period starting with the first oviposition. Therefore, daily fecundity was recalculated using the actual oviposition period when available (16 data points). When no data on post-oviposition period were given (2 data points), longevity was reduced with pre-oviposition period only (indicated in ESME).

Outliers

Preliminary analysis of scatter plots with data points revealed some outliers in the data. When a data point had a distance of more than two standard deviations from the mean at a certain temperature it was omitted from the analysis. In some cases the development rate at 35°C was a clear outlier (for L2, L3, and L (D2, D3, and D5) (Wang

et al. 2009)), which was excluded as well. The study by Lundgren (2009) showed poor performance on soybean leaves (with *Ephestia* food); therefore only the no-plant control was included in the dataset and outliers were excluded. Nedved and Kalushkov (2012) reported low survival for the larval and pupal stages studied (S2, S3, S4, S6) and attributed it to the larvae being grouped during the experiment; therefore their data were also excluded from the dataset. Outliers are marked in the raw data (ESME).

Explanatory variables

To explain variation in life history characteristics reported in the different studies, several variables were selected as factors in the analysis. Unfortunately, not all studies provided an exhaustive list of parameters to describe the experimental setup. Moreover, for the final analysis it was required to lump the factor levels into classes, despite some loss of detail. Below, a description of each factor follows, including explanation of choices made in case of missing values or lumping of data. A summary is given in table 1 of the main text (copy included here).

1. Temperature: The temperature, at which the experiment has been conducted, is used in the analysis as a continuous variable in the range of 14°C to 35°C. When temperature is not or cannot be specified (e.g. in case of field data), the data are excluded.
2. Origin: The origin of the beetles used in the experiment. When the paper does not explicitly state the origin of the beetles, the location of the university has been used as origin. For analysis, data were lumped into two categories: Asian (native range) and non-Asian (invasive range: North America, Europe, South America, and Africa). Ultimately, the studies from South America (1 study) and Africa (1 study) were excluded based on other criteria.
3. Photoperiod: Studies conducted at extreme photoperiods were excluded from the dataset. In case of an unknown photoperiod, it was assumed that the experiment was performed under natural light conditions although some studies were conducted in Asia, where researchers often used extreme photoperiods. Photoperiods were lumped into three categories: short (14–15.5 hours light), long (16–18 hours light), and natural (also including data with unknown photoperiod). Too few studies on larval and pupal survival were performed under natural day conditions. Therefore, for all survival response variables (S1 to S8), except egg survival (S0), data under natural day were excluded from the dataset, and models were fitted with two classes for photoperiod (short and long).
4. Food: In the analysis no distinction was made between aphid species, one of the reasons being that for many aphid species only one data point was available, which hampers the analysis. A classification into aphid and *Ephestia* (also including combined diets of aphids and *Ephestia*) was used for the analysis. For the following response variables very few data with *Ephestia* as food were available, therefore models were fitted that did not contain the factor food: survival L1, L2, L3, L4, and pupa (S1 to S4, S6).
5. Strain: The populations used could either be a reared population or a (recently) field-collected population (incl. subsequent short-term rearing of max. 4 gene-

rations). When the strain type was not clearly stated in the paper, a strain was assigned to the data based on the other information given in the paper.

6. Sex: In case of the response variable longevity (A4) the factor sex was added, as many studies report differences in longevity between sexes. When sex was unknown (four data points that did meet the other criteria), data were excluded.
7. Origin-photoperiod: For survival of L1, L2, L3, L4, larva, and pupa (S1 to S6) the data for factors origin and photoperiod were very confounded. Therefore, these factors were combined into one factor (origin-photoperiod) with three classes: Asian&short, Asian&long, and invasive&long.

In some case more than one data point was retrieved from one study. As conditions within one study presumably are more uniform than between studies, study is modelled as a random effect.

Copy of table 1 *Description of explanatory variables and factors used for model fit. Column 'added' indicates the order in which the explanatory variable were included in model when generating the most complex linear mixed model.*

Factor	Added	Classes	Description
temperature	1	continuous scale	14 to 35°C
origin	2	Asian; invasive	area of origin of ladybirds
photoperiod	3	short; long; natural	photoperiod during experiment: light during 14–15.5 hours, 16–18 hours, or naturally occurring (including unknown photoperiod)
food	4	Aphid; *Ephestia*	food of ladybird[c] used during experiment: aphids only or *Ephestia kuehniella* eggs (with or without aphids)
strain	5	field; reared	source of population: field-collected population (incl. subsequent short-term rearing of max. 4 generations) or laboratory reared population
sex[a]	6	female; male	
origin-photoperiod[b]	A[d]	Asian&short; Asian&long; invasive&long	origin and photoperiod combined, because factors were confounded

[a] *for response variable longevity*
[b] *for response variables survival L1, L2, L3, L4, and pupa*
[c] *of mother in case of eggs, of larva in case of pupa*
[d] *added after temperature and before food if origin and photoperiod were confounded*

Meta-analysis — model selection

For each response variable, first, the most complex linear mixed model was formulated, i.e. all defined factors that were independent from each other were included as long as the number of estimated effects was within the limits the number of data points allowed for. The factors used were origin, photoperiod, food, and strain. In case of small data sets, confounded factors were coded as combination origin-photoperiod. Moreover, temperature was included as continuous explanatory

variable. Each factor could be part of the model as a fixed effect, or additionally in interaction with one of the other factors or with temperature. Study is modelled as a random effect. Subsequently, model selection was performed; all possible less complex models that can be assembled with the factors of the most complex model, were fitted automatically to the data using the R function dredge from the R package MUiMin (version 3.0.2).

Thereafter, the best models were selected with Akaike's Information Criterion (AIC), which is based on the maximum likelihood. Because of small sample sizes, we used the corrected AIC (AICc) (Motulsky and Christopoulos 2004), which is a measure of the relative goodness of fit of the model.

$$\mathrm{AICc} = 2k - 2\mathrm{Log}(L) + \frac{2k(k+1)}{n-k-1}$$

with *Log* denoting the natural logarithm, *L* the maximum likelihood, *k* the number of estimated parameters, and *n* denoting the sample size.

The model with the lowest AICc value is the best, but models with a difference in AICc that is less than 2 are considered as equivalent (Bolker 2008). Within the group of models with ΔAICc < 2 the Ockhams's razor has been used, which is the principle of parsimony. This principle states that among competing hypotheses/models (with the same explanatory power) the one that makes the fewest assumptions should be used. Model selection with AICc is not based on the significance of the individual factors in the model, but it selects the overall best model. As a consequence, the models are presented with the AICc value for the full model, and their relative believe (= AICc weight) within the models that differ less than 2 AICc points (Burnham and Anderson 2002).

$$\textit{AICc weight of model } i = \frac{\exp\left(\frac{-\Delta\mathrm{AICc}_i}{2}\right)}{\sum_{i=1}^{m}\exp\left(\frac{-\Delta\mathrm{AICc}_i}{2}\right)} \quad \textit{with}$$

$$\Delta\mathrm{AICc}_i = \mathrm{AICc}_i - \min_i \mathrm{AICc}_i$$

Linear mixed models with and without temperature were used to model longevity (A4) and rates of development (D0 to D8), pre-oviposition period (A1), and generation time (A2). Fecundity (A3) models included temperature effect in polynomials of order 2 (quadratic), and survival models included temperature effect in polynomials of order 4 (quartic).

For the response variables survival L1, L2, L3, L4, larva, and pupa (S1 to S6) data sets were small and the two factors photoperiod and origin were confounded; thus, no reliable models could be fitted. Therefore, the two factors were combined into one

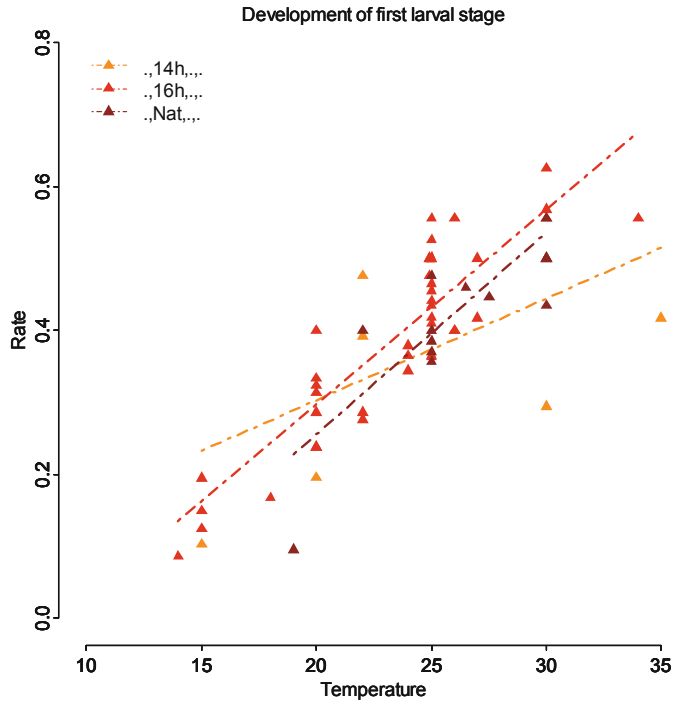
factor with three classes: Asian&short, Asian&long, and invasive&long, reducing the number of explanatory variables, which is preferable with small data sets. The few studies that did not fit in these three classes were excluded from further analysis.

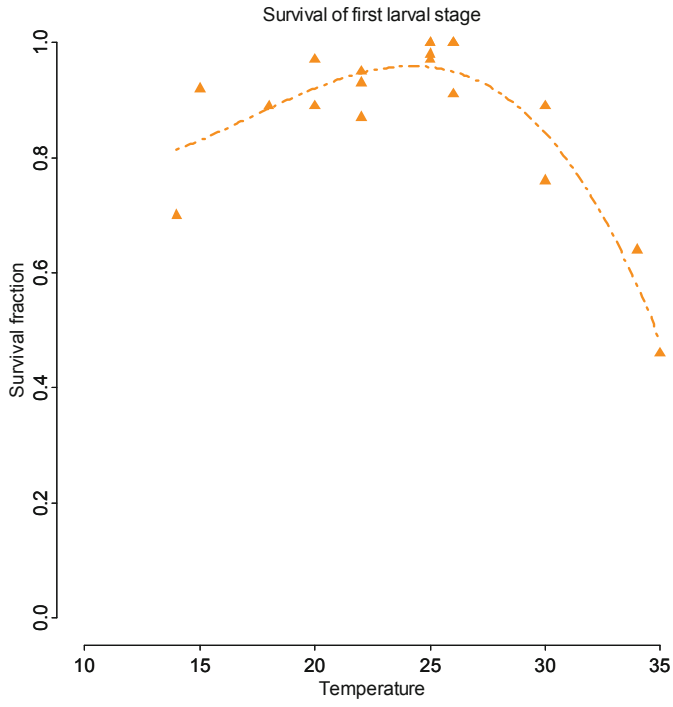
In the analysis, data were weighted with the square root of the number of replicates used to obtain the value for the life history trait. He et al. (1994) did not give a number of replicates in the group that they followed. Therefore, the following values were used: N = 65 for egg development (D0), which is the median number of replicates for egg development; N = 33 for development of larvae, pupa, larva plus pupa, and all immature stages (D5 to D8), which is the average of the median numbers of replicates of each of these response variables as data were determined on the same group of individuals; N = 17 for pre-oviposition period (A1), generation time (A2), fecundity (A3), and longevity (A4), which is equal to half of the number of replicates for development, as these variables were determined on the females only.

When in the course of the experiment the number of replicates decreased due to larval mortality, N was not adjusted accordingly, unless the authors gave separate values for N for each developmental stage. When generation time (A2) was calculated as the sum of immature development time (D8) and pre-oviposition period (A1), N of the latter response variable was used, as pre-oviposition period is usually based on a lower number of individuals. For egg survival (S0) the number of replicates was converted into the number of eggs by either (number of females*total fecundity), or (number of clutches*clutch size), or (number of larvae*sex ratio).

Appendix 2 chapter 8

Figure A1 *Graphs of best, most parsimonious model for development en survival response variables that are not shown in main text. Title of each graph shows for which group the data points are shown and the line is plotted. Data points from literature are represented by symbols. Fitted relationships of the best models, i.e. the most parsimonious model within Δ AICc < 2, are represented by lines that are only shown when data are available for that set of factors. Legends show the combination of factors. Geographic origin: Asia or invasive (EUS). Photoperiod: short (14h), long (16h), or natural (Nat). When no distinction is made between the classes of a factor, that factor is represented by '.'. The x-axis shows temperature starting at 10°C.*


Development of first larval stage
.,14h,.,.
.,16h,.,.
.,Nat,.,.
Rate
0.0
0.2
0.4
0.6
0.8
10
15
20
25
30
35
Temperature


Survival of first larval stage
Survival fraction
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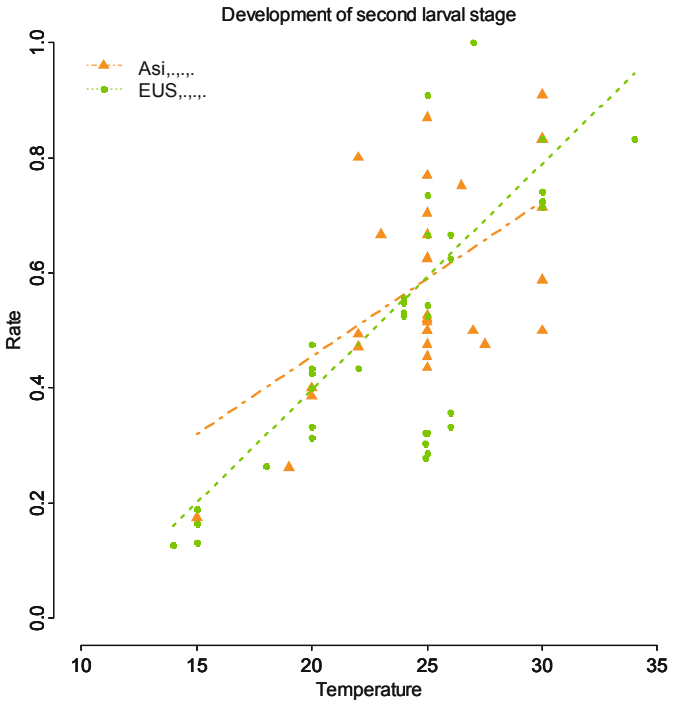
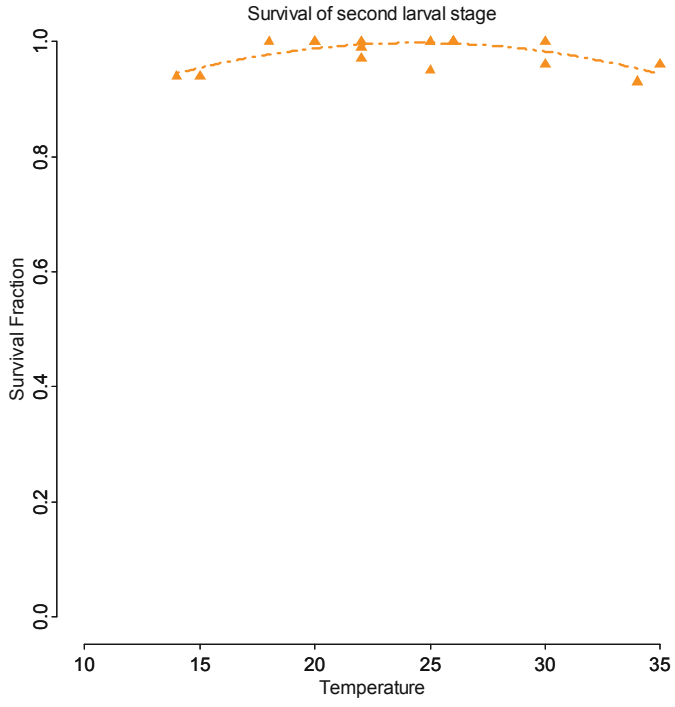
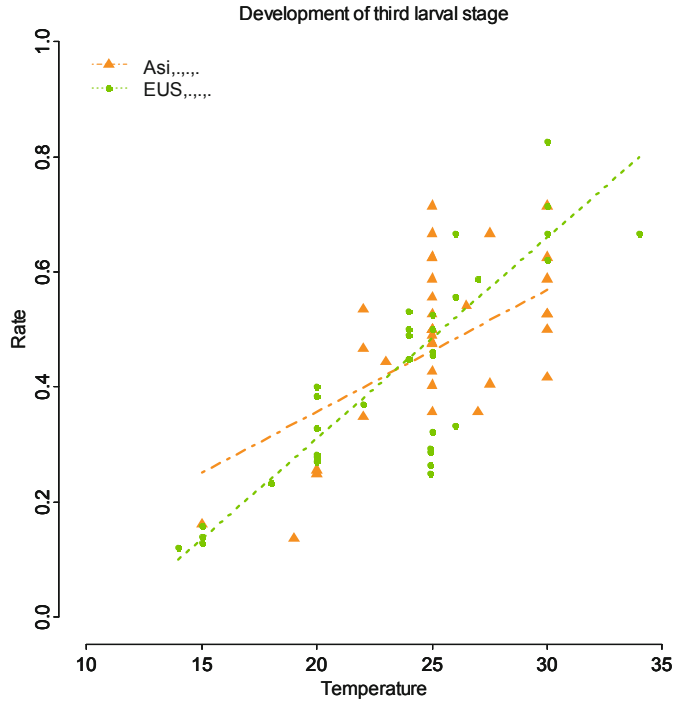
0.2
0.4
0.6
0.8
1.0
10
15
20
25
30
35
Temperature


Development of second larval stage
Asi,.,.,.
EUS,.,.,.
Rate
0.0
0.2
0.4
0.6
0.8
1.0
10
15
20
25
30
35
Temperature


Survival of second larval stage
Survival Fraction
0.0
0.2
0.4
0.6
0.8
1.0
10
15
20
25
30
35
Temperature


Development of third larval stage
Asi,.,.,.
EUS,.,.,.
Rate
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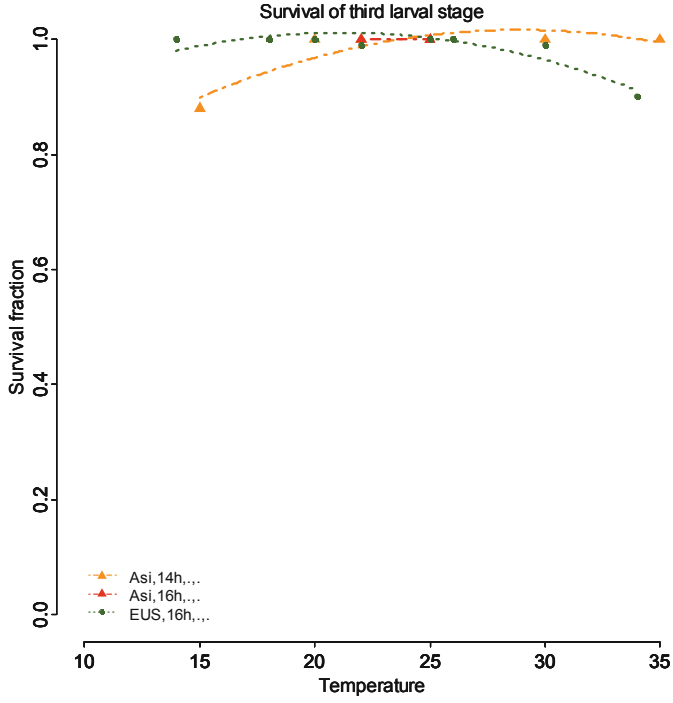
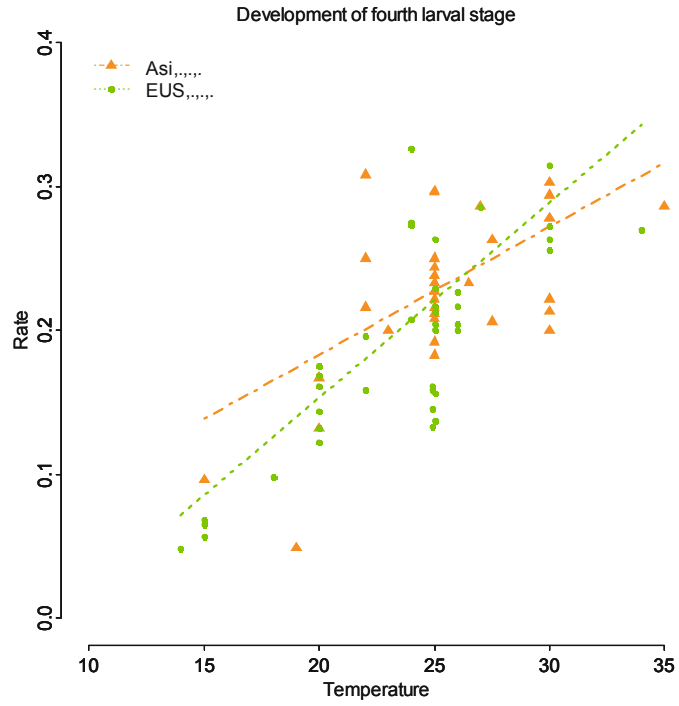
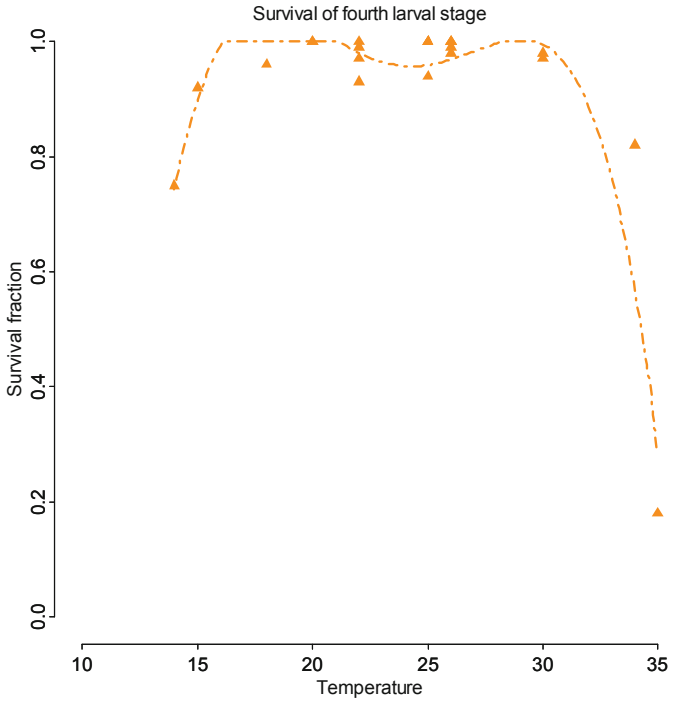
0.2
0.4
0.6
0.8
1.0
10
15
20
25
30
35
Temperature


Survival of third larval stage
Survival fraction
0.0
0.2
0.4
0.6
0.8
1.0
Asi,14h,.,.
Asi,16h,.,.
EUS,16h,.,.
10
15
20
25
30
35
Temperature

8


Development of fourth larval stage
Asi,.,.,.
EUS,.,.,.
Rate
0.0
0.1
0.2
0.3
0.4
10
15
20
25
30
35
Temperature


Survival of fourth larval stage
Survival fraction
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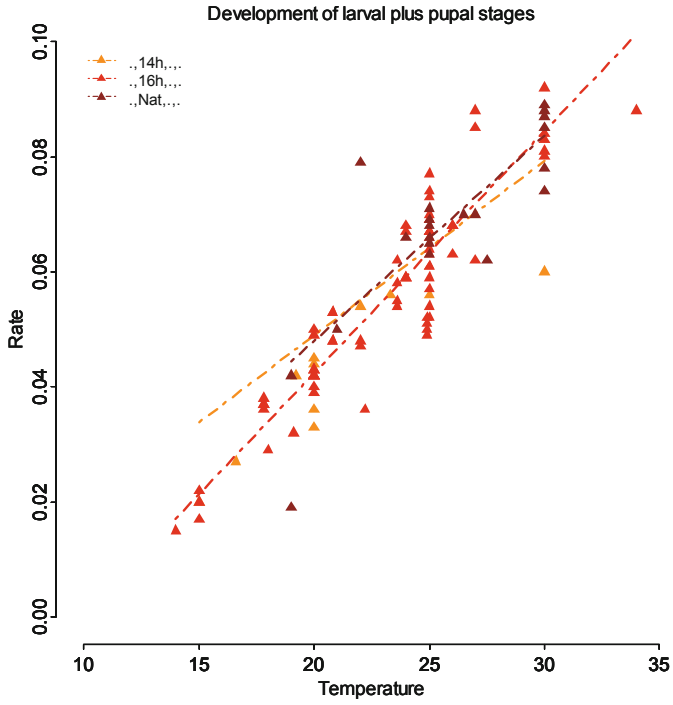
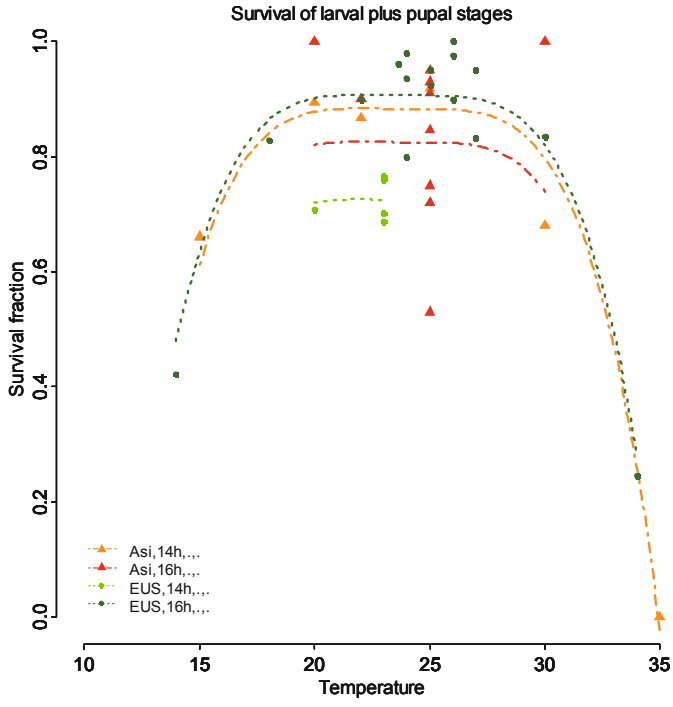
0.2
0.4
0.6
0.8
1.0
10
15
20
25
30
35
Temperature


Development of larval plus pupal stages
.,14h,.,.
.,16h,.,.
.,Nat,.,.
Rate
0.00
0.02
0.04
0.06
0.08
0.10
10
15
20
25
30
35
Temperature


Survival of larval plus pupal stages
Asi,14h,.,.
Asi,16h,.,.
EUS,14h,.,.
EUS,16h,.,.
Survival fraction
0.0
0.2
0.4
0.6
0.8
1.0
10
15
20
25
30
35
Temperature

8

Appendix 3 chapter 8

Table A1 *Average values for development, survival and adult development of* Harmonia axyridis *under extreme photoperiod (0 or 24 hours light), at temperature 23–27°C. N = number of studies.*

		Aphid					Artificial diet				
Response variable	Code	N	Min	Max	Mean (weighted)	SD	N	Min	Max	Mean (weighted)	SD
		Development									
Egg	D0	1	0.25	0.25	0.25	0.00	0				
Larva 1	D1	6	0.37	0.50	0.43	0.05	5	0.19	0.38	0.27	0.07
Larva 2	D2	6	0.50	0.81	0.66	0.11	5	0.27	0.37	0.32	0.04
Larva 3	D3	6	0.33	0.70	0.57	0.12	5	0.19	0.36	0.26	0.08
Larva 4	D4	6	0.17	0.26	0.23	0.03	5	0.08	0.18	0.14	0.04
Pupa	D5	5	0.18	0.21	0.20	0.01	1	0.19	0.19	0.19	0.00
Larva	D6	6	0.08	0.11	0.10	0.01	6	0.04	0.09	0.06	0.02
Larva+pupa	D7	7	0.05	0.07	0.06	0.01	48	0.03	0.06	0.05	0.00
Immature	D8	1	0.04	0.04	0.04	0.00	1	0.05	0.05	0.045	0.00
		Survival									
Egg	S0	0					6	0.43	0.80	0.54	0.11
Larva 1	S1	3	0.83	0.97	0.94	0.05	6	0.10	0.95	0.70	0.31
Larva 2	S2	3	0.90	1.00	0.97	0.03	6	0.65	1.00	0.86	0.13
Larva 3	S3	3	0.89	0.97	0.96	0.03	5	0.00	1.00	0.62	0.35
Larva 4	S4	3	1.00	1.00	1.0	0.00	17	0.25	1.00	0.76	0.24
Pupa	S5	3	1.00	1.00	1.00	0.00	16	0.33	1.00	0.72	0.25
Larva	S6	3	0.67	0.92	0.88	0.09	20	0.05	1.00	0.46	0.30
Larva+pupa	S7	4	0.03	0.92	0.53	0.43	57	0.05	1.00	0.41	0.21
Immature	S8	2	0.17	0.18	0.18	0.01	10	0.00	0.34	0.14	0.11
		Adult									
Pre-ovi	A1	0					2	0.05	0.10	0.07	0.03
Gen Time	A2	1	0.03	0.03	0.03	0.01	0				
Daily fec	A3	1	16.70	16.70	16.70	0.00	6	1.13	20.70	10.11	3.89
Longevity	A4	1	32.20	32.20	32.20	0.00	0				

Values based on data retrieved from the following studies: Dmitriew and Rowe 2011; Hongo and Obayashi 1997; Hukusima and Itoh 1976; Matsuka and Okada 1975; Niijima et al. 1977; 1997; Niijima and Takahashi 1980; Okada et al. 1971; 1973; 1978; Okada and Matsuka 1973; Okamoto 1978; Tedders and Schaefer 1994

Appendix 4 chapter 8

Life cycle parameters other species

Table A2 *Overview of literature data on life cycle and reproduction parameters for* Harmonia axyridis*,* Adalia bipunctata*,* Coccinella septempunctata*, and* Propylea quatuordecimpunctata*.* a*: Threshold temperature and thermal constant;* b*: R_0, r_m, and mean generation time Birch;* c*: generations/year, sex ratio, and field mortality. References are given by a number between brackets; complete list of references is given below the table. ISO-3661-1 codes are used to indicate country of origin beetles.*

a

Threshold immature development			Thermal constant immature development (degree-days)		
Europe	**North America**	**Asia**	**Europe**	**North America**	**Asia**
			Harmonia axyridis		
11.2 [1][a]	11.2 [4]		258.3 [1][a]	267 [4]	330.4 [5][b]
10.8 [1][a]			243.6 [1][a]		
10.5 [2][a]			231.3 [2][a]		
10.6 [3][a]					
			Adalia bipunctata		
10.06 [6][ac]	9 [9]	6.3 [11]	267.9 [6] [ac]	262.8 [9]	322.6 [11]
9.39 [6][acd]	6.8 [10][e]		266.27 [6] [acd]	269 [10][e]	
9.2 [7] in [6]			251.81 [7] in [6]		
8.5 [8] in [6]			244.8 [8] in [6]		
			Coccinella septempunctata		
10.5 [12]	12.1 [9]	11.1 [17]	281.5 [12]	196.8 [9]	209.9[17]
12.4 [13] in [14]	13.7 [16] in [15]		206 [13] in [14]	191.0 [16] in [15]	
11.3 [Alan (1980) in 15]			320.0 [Alan (1980) in 15]		
			Propylea quatuordecimpunctata		
10.2 [18][c]	9.9 [19][f]		218 [18][c]	207 [19][f]	
10.3 [19][f]			198.5 [20]		
9.9 [19][f]			213 [19][f]		
11.3 [20][a]			217 [19][f]		
11.6 4 [13] in [14]			164 [13] in [14]		
15 [Quilici (1981) in 20]			144 [Quilici (1981) in 20]		

b

R_0			r_m			Mean generation Time (Birch)		
Europe	**North America**	**Asia**	**Europe**	**North America**	**Asia**	**Europe**	**North America**	**Asia**
				Harmonia axyridis				
26.27 [21] 25°C[ag]	322.66 [22] 15°C[h]	176.12 [5] 15°C	0.089 [21] 25°C[ag]	0.12 [22] 15°C[h]	0.06 [5] 15°C	38.81 [21]	46.36 [22] 15°C[h]	89.06 [5] 15°C
	243.09 [22] 20°C[h]	299.28 [23] 20°C[i]	0.15 [25] [ade]	0.13 [22] 20°C[h]	0.099 [23] 20°C[i]	25°C[ag]	42.61 [22] 20°C[h]	90.95 [23] 20°C[i]
	278.03 [22] 25°C[h]	81.24 [23] 20°C[i]		0.238 [26] 24°C	0.073 [23] 20°C[i]		39.48[22] 25°C[h]	77.47 [23] 20°C[i]
		53.06 [23] 20°C[i]		0.113 [27] 24°C[d]	0.093 [23] 20°C[i]			45.34 [23] 20°C[i]
		16.86 [23] 20°C[i]		0.14 [22] 25°C[h]	0.04 [23] 20°C[i]			79.98 [23] 20°C[i]
		537.95 [5] 20°C			0.09 [5] 20°C			75.51 [5] 20°C
		98.89 [5] 25°C			0.148 [24] 25°C			58.47 [5] 25°C
		382.65 [24] 25°C			0.15 [5] 25°C			44.01 [5] 30°C
		417.01 [5] 30°C			0.13 [5] 30°C			
				Adalia bipunctata				
337.77 [28] 19°C[a]		245.5 [29] 27°C[a]	0.1077 [28] 19°C[a]		0.178 [29] 27°C[a]	54.07 [28] 19°C[a]		
344.11 [28] 19°C[a]		383 [29] 27°C[a]	0.0967 [28] 19°C[a]		0.194 [29] 27°C[a]	60.39 [28] 19°C[a]		
141.56 [28]		233.1 [29] 27°C[aj]	0.0862 [28] 19°C[ad]		0.173 [29] 27°C[aj]	58.26 [28] 19°C[a]		
19°C[ad]		217 [29] 27°C[aj]	0.1425 [28] 23°C[a]		0.166 [29] 27°C[aj]	37.91 [28] 23°C[a]		
221.7 [28] 23°C[a]			0.1516 [28] 23°C[a]			38.75 [28] 23°C[a]		
355.79 [28] 23°C[a]			0.1303 [28] 23°C[ad]			38.1 [28] 23°C[a]		
133.86 [28]			0.081 [21] 25°C[g]			40.06 [21] 25°C[g]		
23°C[ad]			0.1895 [28] 27°C[a]			28.63 [28] 27°C[a]		
18.49 [21] 25°C[g]			0.1641 [28] 27°C[a]			30.9 [28] 27°C[a]		
227.71 [28] 27°C[a]			0.1369 [28] 27°C[ad]			25.5 [28] 27°C[a]		
159.66 [28] 27°C[a]			0.129 [25][de]					
31.05 [28] 27°C[ad]								
				Coccinella septempunctata				
54.9 [30] 23°C	73 [33] 25°C	52.8 [34] 25°C[k]	0.071 [30] 23°C	0.085 [33] 25°C	0.086 [34] 25°C[k]	56.8 [30] 23°C	50 [33] 25°C	20 [34] 25°C[k]
1004.1 [31] 25°C	559.6 [32] 26°C	575.5 [35] 25°C	0.118 [31] 25°C	0.17 [32] 26°C	0.145 [35] 25°C	58.6 [31] 25°C	31.9 [32] 26°C	43.8 [35] 25°C
563.5 [32] 26°C	663.9 [32] 26°C		0.18 [32] 26°C	0.2 [32] 26°C		34 [32] 26°C	37.2 [32] 26°C	
567.6 [32] 26°C			0.19 [32] 26°C			35.4[32] 26°C		
				Propylea quatuordecimpunctata				
375.1 [31] 25°C	148.9 [19] 26°C[f]		0.166 [31] 25°C	0.215 [26] 24°C		35.7 [31] 25°C	37.1 [19] 26°C[f]	
149.9 [19] 26°C[f]			0.15 [19] 26°C[f]	0.14 [19] 26°C[f]		33.1 [19] 26°C[f]		
189.5 [19] 26°C[f]			0.15 [19] 26°C[f]			33 [19] 26°C[f]		
			0.069 [25] [de]					

C

Generations /year	Sex ratio (%female)	Field mortality
Harmonia axyridis		
2–3 overlapping, DK [36]	52.2, FR [45] 18°C[d]	62–90% [caged, 53]
2, UK [37]	52.5, UK [45] 18°C[d]	0–55.6% [caged, 54]
2, NL (PS van Wielink pers. obs.)	49.6, FR [45] 18°C[ad]	98.9%, 1993 [55]
3, NL (AJM Loomans pers. obs.)	41.9–44.8, CN [23] 20°C	92.9%, 1994 [55]
2, BE [38]	53, FR [46] 22°C[ad]	99.18%, 1987 [56]
2, FR [39]	49, DE [47] 23°C[a]	98.32%, 1987 [57]
4, IT [25]	40, DE [47] 23°C[ad]	99.01%, 1988 [57]
2, Minnesota, USA [40]	54, FR [45] 24°C[d]	99.1%, 1993 [mort larva, 58]
2–3, Oregon, USA [41]	38.3, UK [45] 24°C[d]	24.5%, 1994 [mort larva, 58]
2–8, CN [42]	54, FR [45]24°C[ad]	98.0%, 1995 [mort larva, 58]
2–8, CN [43]	48.6, CA [26] 24°C	96.6%, 1996 [mort larva, 58]
4, GR [44]	39, IT [21] 25°C[a]	
	50.7–61.8, Azores, PT [48] 25°C[ad]	
	43–54, Azores, PT [49] 25°C[ad]	
	49, Azores, PT [49] 25°C[a]	
	29, CZ [50] 25°C	
	52, CZ [50] 25°C	
	61, CZ [50] 25°C	
	38, CZ [50] 25°C[d]	
	48, CZ [50] 25°C[d]	
	82, CZ [50] 25°C[d]	
	52, USA [51] 27°C[d]	
	54.8, Asia [52] 27°C	
	35.1, FR [45] 30°C[d]	
	15.6, UK [45] 30°C[d]	
	48, FR [45] 30°C[ad]	
Adalia bipunctata		
2, central EUR [59]	54, BE [28] 19°C[ad]	23.1–88.9% [caged, 54]
1, North & West EUR [59]	60–63, BE [28] 19°C[a]	
1–2, NL [60]	55, BE [28] 23°C[a]	
3, BE [61]	58, BE [28] 23°C[ad]	
1 partially 2, UK [62]	48–49, BE [28] 27°C[a]	
2–3, Ithaca, USA [19]	54, BE [28] 27°C[ad]	
4, IT, [25]	57, NL [65] 20°C	
4, Berlin, DE, [63]	55, PL [64] field obs.	
3, PL [64]		
1, North SE [59]		
2, South SE [59]		

[b] *values for egg, larva and pupa summed*
[c] *only values for linear model shown*
[i] *four different colour morphs*
[d] *fed with* Ephestia
[g] *larvae reared on* Ephestia*, adults reared on aphids*
[a] *reared population*
[e] *overwintering population*
[f] *the author has confirmed that pre-imaginal means from egg to adult emergence*
[j] *aphids reared on non-host plant*
[k] *Saoedi Arabia*
[h] *South America*

c-continued

Generations /year	Sex ratio (%female)	Field mortality
Coccinella septempunctata		
1 sometimes 2, EUR [66]	33.3, GR [12] 14°C	61.7%, 1992, [caged, mort larva+pupa, 69]
1–2, GR [30]	47.8, GR [12] 17°C	100%, 1993, [caged, mort larva+pupa, 69]
47–80% second generation, USA [67]	50, GR [12] 20°C	60.8%, 1992, [with *C. maculata*, 69]
	34.8, GR [12] 23°C	100%, 1993, [with *C. maculata*, 69]
1 sometimes partially 2, UK [68]	47, FR [32] 26°	0%, 1976 [70]
1 partially 2, UK [62]	50–52, US [32] 26°C	0%, 1976 [70]
	55, UA [32] 26°C	0%, 1977[70]
		62%, 1977 [70]
		95%, 1978 [70]
		22%, 1978 [70]
		subspecies brucki:
		100%, 1993, [55]
		95.4%, 1994, [55]
		99.9%, 1993, [mort larva, 58]
		81.3%, 1994, [mort larva, 58]
		99.7%, 1995, [mort larva, 58]
		85.6%, 1996, [mort larva, 58]
Propylea quatuordecimpunctata		
56 & 80% second generation, Europe [67]	15–41, TU [71] 20°C	
	26.1–40, UA [71] 20°C	
83% second generation, USA [67]	31.8–42, [71] 20°C	
1 partially 2, UK [62]	57.6, CA [26] 24°C	
	41, FR [19] 26°C	
	42, TU [19] 26°C	
	42, CA [19] 26°C	
	44, FR [19] 14°–30°C pooled	
	39, TU [19] 14°–30°C pooled	
	51, CA [19] 14°–30°C pooled	

References: [1] (Stathas et al. 2011), [2] (Schanderl et al. 1985), [3] (Brun 1993), [4] (LaMana and Miller 1998), [5] (Wang et al. 2009), [6] (Jalali et al. 2010), [7] (Schuder et al. 2004), [8] (Ellingsen 1969), [9] (Obrycki and Tauber 1981), [10] (Obrycki et al. 1983), [11] (Sakuratani et al. 2000), [12] (Katsarou et al. 2005), [13] (Hodek 1958), [14] (Baumgärtner et al. 1987), [15] (Hodek and Honek 1996), [16] (Butler 1982), [17] (Xia et al. 1999), [18] (Papanikolaou et al. 2013), [19] (Obrycki et al. 1993), [20] (Hemptinne et al. 1988), [21] (Lanzoni et al. 2004), [22] (Castro et al. 2011), [23] (Kuang and Xiao 1995), [24] (Zhao et al. 2012), [25] (Bazzocchi et al. 2004), [26] (Mignault et al. 2006), [27] (Michaud and Grant 2003), [28] (Jalali et al. 2009), [29] (Jalali and Michaud 2012), [30] (Zarpas et al. 2007), [31] (Kontodimas et al. 2008), [32] (Phoofolo and Obrycki 1995), [33] (Stark et al. 2004), [34] (ElHag and Zaitoon 1996), [35] (Kawauchi 1985), [36] (Steenberg and Harding 2009b), [37] (Brown et al. 2008b), [38] (Adriaens et al. 2008), [39] (Ongagna and Iperti 1994), [40] (Koch and Hutchison 2003), [41] (LaMana and Miller 1996), [42] (Wang et al. 2007), [43] (Wang and Shen 2002), [44] (Katsoyannos et al. 1997a), [45] (Lombaert et al. 2008), [46] (Tourniaire et al. 1999), [47] (Kögel et al. 2012a), [48] (Serpa et al. 2003), [49] (Schanderl et al. 1988), [50] (Nedved and Kalushkov 2012), [51] (Lundgren et al. 2009), [52] (McClure 1987), [53] (Hoogendoorn and Heimpel 2004), [54] chapter 5, [55] (Yasuda and Shinya 1997), [56] (Osawa 1992b), [57] (Osawa 1993), [58] (Kindlmann et al. 2000), [59] (Webberley et al. 2006b), [60] (Brakefield 1984), [61] (Hemptinne and Naisse 1988), [62] (Majerus 1994), [63] (Timofeeff-Ressovsky and Svirezhev 1966), [64] (Webberley et al. 2006a), [65] (Ottenheim et al. 1992), [66] (Hodek 2012b), [67] (Phoofolo and Obrycki 2000), [68] (Zhou and Carter 1992), [69] (Obrycki et al. 1998), [70] (Basedow 1982), [71] (Michels and Flanders 1992).

Appendix 5 chapter 8
References used in Electronic Supplementary Materials

References used in ESMD — Life history data native species

Baumgärtner et al. 1987; Blackman 1967; Bonte et al. 2010; De Clercq et al. 2005; Dixon and Guo 1993; Ferrer et al. 2008; Francis et al. 2001; Ghanim et al. 1984; Hämäläinen et al. 1975; Hemptinne et al. 1988; Iablokoff-Khnzorian 1982; Jalali et al. 2009; Jalali et al. 2010; Kalushkov 1994; Kalushkov and Hodek 2004; 2005; Katsarou et al. 2005; Kontodimas et al. 2008; Lanzoni et al. 2004; Michels and Flanders 1992; Obrycki et al. 1993; Papanikolaou et al. 2013; Phoofolo and Obrycki 1995; 2000; Schuder et al. 2004; Sundby 1966; Ware et al. 2008; Yasuda and Dixon 2002; Zarpas et al. 2007

References used in ESME— Raw data

Abdel-Salam and Abdel-Baky 2001; Agarwala et al. 2008; Agarwala and Bhowmik 2011; Bazzocchi et al. 2004; Berkvens et al. 2008a; 2008b; Brun 1993; Butin et al. 2004; Chen et al. 2012; Cottrell 2004; Dixon and Agarwala 2002; Dmitriew and Rowe 2007; Dong et al. 2001; Evans and Gunther 2005; Facon et al. 2011a; Ferran et al. 1998; Galvan et al. 2005; Grill et al. 1997; Grill and Moore 1998; Guo and Wan 2001; He et al. 1994; Hongo and Obayashi 1997; Hukusima and Kamei 1970; Hukusima and Ohwaki 1972; Iablokoff-Khnzorian 1982; Jing et al. 2002; Kalaskar and Evans 2001; Katsoyannos et al. 1997a; Kawai 1978; Kawauchi 1979; Kim and Choi 1985; Kindlmann et al. 2000; Kögel et al. 2012a; 2013; Kraiss and Cullen 2008a; 2008b; Krengel et al. 2012; Kuang and Xiao 1995; Labrie et al. 2006; LaMana and Miller 1996; 1998; Lanzoni et al. 2004; Lombaert et al. 2008; Lundgren et al. 2009; Matsuka and Niijima 1985; Matsuka and Takahashi 1977; McClure 1987; Michaud 2002b; 2003; Michaud and Grant 2003; 2004; Michaud and Olsen 2004; Mignault et al. 2006; Moser and Obrycki 2009; Nakamura et al. 2006; Nedved and Kalushkov 2012; Niijima et al. 1986; Nimmo and Tipping 2009; Obata 1987; Okada et al. 1972; Okamoto 1978; Ongagna et al. 1993; Ongagna and Iperti 1994; Osawa 1989; 1992a; 1992b; 1992c; 1993; 2002; 2003; Osawa and Ohashi 2008; Phoofolo and Obrycki 1998; Reznik and Vaghina 2011; 2013; Rieder et al. 2008; Saini 2004; Saito and Bjornson 2008; Sakurai et al. 1993; Santi and Maini 2007; Sato and Dixon 2004; Schanderl et al. 1985; 1988; 1995; Seko and Miura 2009; Seko et al. 2012; Seo et al. 2008; Serpa et al. 2003; Sighinolfi 2005; Sighinolfi et al. 2008; Soares et al. 2001; 2004; Specty et al. 2003; Stathas et al. 2001; 2011; Stuart et al. 2002; Sun et al. 1996; Takahashi 1986; 1989; Tan 1933; Tan and Li 1932; Tourniaire et al. 1999; Tsaganou et al. 2004; Turgeon et al. 2011; Ueno 2003; Ungerova et al. 2010; Wang 1986; Wang and Shen 2002; Wang et al. 2007; Wang et al. 2009; Ware et al. 2009; Yang et al. 2003; Yasuda and Kimura 2001; Yasuda and Ohnuma 1999; Yasuda and Shinya 1997; Yuan et al. 1994; Zhang et al. 2009; Zhao et al. 2012; Zhu et al. 1996.

9

General discussion

C Lidwien Raak-van den Berg

The general aim of my thesis research was to determine the causes and consequences of the establishment and rapid spread of *H. axyridis* in the Netherlands. In this chapter I will take my research questions as a starting point to summarise my research results. I will then go on to discuss the factors that may explain the invasiveness of *H. axyridis* and whether *H. axyridis* will completely displace native species. Finally, I will put forward suggestions for future research.

What are the causes and consequences of the establishment of *H. axyridis* in the Netherlands?

Below, I give the main points of this research by means of the four research questions formulated in the introductory chapter.

What is the life history of *H. axyridis* and does it help to understand the causes of its rapid establishment and spread in the Netherlands?

In the field *H. axyridis* has a short diapause compared to native species (chapter 2 and 3). This has the advantage that, first, the diapause prevents the ladybirds from emerging "too early" when temperatures rise temporarily in mid-winter, and, second, that beetles can become active rapidly when they are in post-diapause stage when spring arrives. However, the developmental threshold of *H. axyridis* is similar to or higher than that of native species and phenological data do not suggest that *H. axyridis* is active earlier than native species (chapters 2 and 3).

Overwintering survival of *H. axyridis* under field conditions is high compared to native species (chapter 4). *Harmonia axyridis* avoids extreme environmental conditions by hiding in cracks and crevices of buildings and other structures (Nalepa et al. 2005; Wang et al. 2011); it cannot survive in leaf litter (chapter 4), which is the overwintering habitat of native *C. septempunctata* (Hodek 2012b). Higher overwintering survival of the exotic species compared to native species may result in larger populations in spring, giving an advantage in population growth, which may lead to native species being outnumbered.

Chapter 5 shows that larval survival of *H. axyridis* in the field in spring and summer can be high but strongly depends on climatic conditions. Generally, immature development of *H. axyridis* was slower, but survival was higher than that of native *A. bipunctata*. Slower development might result in a lower number of generations per year when there are no other constraints determining the number of generations (i.e. food limitation, obligatory diapause), but this might be counteracted by higher survival resulting in a larger adult population per generation for *H. axyridis.*

In chapter 8 I compared life history characteristics modelled for *H. axyridis* with those of native species retrieved from literature. This resulted in the following conclusions: development of *H. axyridis* is slower than that of native species, but *H. axyridis* lives longer, lays more eggs, and has more generations per year than native species. All characteristics, except rate of development, result in an expected faster population growth of *H. axyridis* compared with native species and are likely to contribute to the invasion success of *H. axyridis*.

How does *H. axyridis* interact with native ladybird species and what are the consequences of its establishment?

Under field conditions with high food-availability, I found no effect of the presence of *H. axyridis* on the survival, development, weight, and size of the native species *A. bipunctata* (chapter 5), and I speculate that the same holds for other native species. However, under field conditions aphid colonies are relatively short-lived (Dixon 1998) — quick growth followed by a rapid decline — which causes situations of prey scarcity and intraguild predation may occur (chapter 6). I found that under semi-field conditions (i.e. small trees in cages under field conditions) the contact frequency between ladybird species is much lower than observed under laboratory conditions. But as has been found in the laboratory, *H. axyridis* is the strongest intraguild predator, and its larvae are the winner in contacts with larvae of the native species *A. bipunctata* and *C. septempunctata*.

In general, i.e. independent of the generation, *H. axyridis* is known to arrive late at aphid colonies, compared to the aphid-peak and to the arrival of native species (Takahashi 1989; Yasuda and Shinya 1997; Kindlmann et al. 2000; Jansen and Hautier 2008; Vandereycken et al. 2013a), and in combination with its slow immature development (chapter 5) this often results in larval development not yet being completed when aphid colonies collapse (Osawa 1992b; Jansen and Hautier 2008; Ameixa et al. 2013). Yet, *H. axyridis* is a strong intraguild predator and can feed and complete its development on eggs, larvae, and pupae of other ladybirds (Yasuda and Ohnuma 1999; Pell et al. 2008; Ware et al. 2009), so its slow immature development is not hampered by unavailability of sufficient food. Strong intraguild predation by *H. axyridis* is thus another characteristic contributing to the explanation of its invasion success.

What is the biology in the invasive range of *H. axyridis* compared with that in their area of origin: are there differences that might explain its invasiveness in Europe?

In its native range in China, *H. axyridis* has two to eight generations per year (Wang and Shen 2002; Wang et al. 2007) and two to four in Japan (Tanigishi 1976; Osawa 2011), depending on climate. In central Japan, *H. axyridis* aestivates in quiescence during mid-summer in leaf-shelters on trees, which is suggested to be an adaptation to survive high temperatures (Osawa 2011). Depending on the local climate, hibernation starts between October and December, lasts three to six months, and emergence occurs in March and April (Tanigishi 1976; Osawa 2011; Wang et al. 2011). In the invasive range in Europe, the life cycle is comparable, except that, to the best of my knowledge, so far no summer quiescence has been reported, and there is a maximum of four generations per year in the south (Katsoyannos et al. 1997a; Bazzocchi et al. 2004). Hibernation in Europe starts in October/November and lasts until March (chapter 4).

In chapter 8 I carried out a meta-analysis and compared available literature data on 22 life history characteristics. Although we found differences between Asian and invasive populations of *H. axyridis*, the greatest differences in development rate were

observed at temperatures above 24°C. No differences were found at temperatures characteristic for spring and summer in northwestern Europe. This indicates that the invasion success of *H. axyridis* cannot be attributed to rapid evolution or hybridisation of genetically different populations. In this meta-analysis only laboratory studies have been compared. Laboratory studies can give valuable insights in the life history of species and in differences between species. In the field, however, many other factors may determine the actual performance of species. In the meta-analysis no obvious differences were observed between native Asian and invasive populations of *H. axyridis* that might be an explanation for its invasiveness.

Which natural enemies attack *H. axyridis* and might be used to control the ladybird in Europe?

The Enemy Release Hypothesis (Roy et al. 2011a) states that an exotic species introduced to a new range may have an advantage over native species because it experiences reduced impacts from natural enemies as it invaded 1) without or with few of its native natural enemies; and 2) the natural enemies in the invaded area are not (yet) effectively attacking the exotic species. Information on natural enemies in the native range of *H. axyridis* is scarce. Infection is reported to be higher in the native area than in the invasive range. In the latter, initially very few natural enemies were found that attacked and could develop on *H. axyridi*s (Roy et al. 2011b). This was partly attributed to the strong chemical and behavioural defence of *H. axyridis* (Firlej et al. 2010; 2012; Sloggett et al. 2011).

I studied which natural enemies attacked *H. axyridis* in the Netherlands and when they started to attack. In this study, the fungus *H. virescens* was first detected in 2010 and locally infected up to 56% of the ladybirds, making it the most frequently occurring natural enemy in that year. The nematode *P. bifurcatus*, the second most common natural enemy, has been observed in three successive years and rates were highest in the final year. Nematode infection is negatively associated with reproduction of *H. axyridis.* This is the first evidence that natural enemies are starting to use *H. axyridis* as host. If future studies show that nematode infection indeed causes a failure to reproduce, nematodes might be a useful species to use for control of *H. axyridis.* The effect of the fungus on *H. axyridis* is not very clear yet. Other natural enemies (parasitoids and mites) are scarce and thus play as yet a very limited role in the reduction of *H. axyridis* populations.

What factors may explain invasiveness of *H. axyridis*?

Invasiveness is 'the degree to which a species is able to reproduce, spread from its place of introduction, and establish in new locations' (Rejmánek 2011). Literature gives a wide array of characteristics that are associated with the success of invasive alien species, such as high growth rate, large climate and environmental tolerance, short generation time, good reproduction, small egg size, good dispersal, high capacity for uni-parental reproduction, absence of hatching requirements, high competitive ability, and ability to escape or survive natural enemies (Whitney and Gabler 2008). However, these characteristics are very general and not all species showing such characteristics become invaders while other species not having such characteristics have been shown

to be invasive (Simberloff 2013). This is probably the case because many other factors influence the invasion process e.g. introduction effort or biotic resistance of the receiving habitat (Clout and Williams 2009; Rejmánek 2011; Simberloff 2013).

Engelkes and Mills (2011) sketched a conceptual framework to understand invasions of, among others, predatory insects. The invasion process of predators can be seen as three sequential stages that are separated by a transition or barrier that must be overcome: arrival, establishment, and spread. Each stage has other factors that contribute to success. In the arrival stage, a species is transported into a new region, most often as the result of human activity – either deliberate or accidental. Success in the subsequent stage of establishment is affected by the environment and by propagule pressure. An alien species can establish when sufficient resources, such as suitable food, are available as well as enemy-free space. A higher propagule pressure, i.e. a higher frequency and/or number of individuals, increases the chance of establishment. Spread, the final stage, occurs when the alien population increases and disperses. Habitat breadth, diet breadth, short generation time, and reproductive advantage over native species are important biological characteristics for spread, especially in combination with potential for rapid adaptation. Spread is also influenced by biological interactions such as a better ability to compete for resources and to defend itself against enemies compared to native species.

My research points at several characteristics that influence the invasion success of *H. axyridis.* The *H. axyridis* case meets a number of the Engelkes and Mills-criteria (2011). Arrival of *H. axyridis* has been facilitated by humans having deliberately introduced the ladybird in many new geographic regions as biological control agent (Brown et al. 2008a; 2011b) and maybe accidentally as well via harbours (Day et al. 1994).

In the establishment stage propagule pressure was increased by repeated introductions, and as a result invasive *H. axyridis* populations still have a large genetic variability (Grill et al. 1997; Krafsur et al. 2005). Environmental characteristics of the invasive range were no constraint for *H. axyridis* as it is able to develop and reproduce under a broad range of conditions (Evans et al. 2011); has a high phenotypic plasticity (Grill et al. 1997; Lombaert et al. 2008); and during overwintering *H. axyridis* forms aggregations in sheltered locations, avoiding exposure to unsuitable environmental conditions (Labrie et al. 2008). The ladybird is eurytopic and polyphagous, which allows it to forage in many habitats and to find non-aphid prey easily when aphids are scarce (Brown et al. 2011b; Hodek et al. 2012; Vandereycken et al. 2012; 2013a). It can even live on pollen (Berkvens et al. 2008a), sugars (Galvan et al. 2008), and on immature stages of conspecific and heterospecific ladybirds (Yasuda and Ohnuma 1999; Cottrell 2004; Sato and Dixon 2004; Sato et al. 2008; Ware et al. 2009). Furthermore, it is multivoltine and has a reproductive advantage over native species, with low larval mortality (chapters 5 and 8) and high fecundity (chapter 8).

Spread in the new area has been stimulated by the biological characteristics mentioned above and by the better ability to compete and to defend itself than native species, but also by the many introductions for biological control of aphids. Within the aphidophagous guild *H. axyridis* is a strong intraguild predator (chapter 6). *Harmonia axyridis* is also better defended by chemical deterrence (Sloggett et al. 2011) and

morphological structure (larval dorsal spines) (Hautier et al. 2010), and it has a greater ability to escape from attack (chapter 6, Firlej et al. 2010) than native species. In addition, during the early phases of the invasion process, very few natural enemies attacked *H. axyridis* (chapter 7). *Harmonia axyridis* has strong dispersal ability as it is a strong and active flyer during summer (Jeffries et al. 2013) as well as before hibernation (e.g. see Obata 1986a; Hodek et al. 1993; Osawa 2000). During invasion it has spread at rates estimated between 100 and 500 km per year (Brown et al. 2011b). Although in our meta-analysis we found no obvious indication that *H. axyridis* had rapidly adapted during invasion, Lombaert et al. (2014) show that several traits related to dispersal have rapidly evolved in less than a decade, which suggests that for some traits the potential to rapidly evolve is present in *H. axyridis*.

Finally, the large size of *H. axyridis* compared to native species is also often mentioned as an important invasion characteristic. It is not the size *per se* that is advantageous, but it is associated with dispersal ability (Hemptinne et al. 2012), reproductive capacity (Kajita and Evans 2010), and intraguild predation strength (Felix and Soares 2004; Ware and Majerus 2008).

The successful invasion of *H. axyridis* in the Netherlands can be explained by the combination of characteristics mentioned above.

Will exotic *H. axyridis* completely displace native species?

Diversity and numbers of ladybird species in Asia appear to be quite stable (Pell et al. 2008; Alhmedi et al. 2010; Osawa 2011). Nevertheless, in Japan Osawa (2011) reported a negative relationship between *H. axyridis*' relative abundance and biodiversity of ladybird species. He attributed this effect primarily to *H. axyridis* both being the dominant intraguild predator species and having a wider habitat range than native species. As a result, *H. axyridis* has a higher relative abundance in unsuitable habitats that are utilised less by the other species, and, as Osawa (2011) states, *'*relative and absolute high abundance of *H. axyridis* did not decrease the density of the coexisting aphidophagous ladybirds per se'.

Several authors (Yasuda and Shinya 1997; Burgio et al. 2002; Dixon 2007; Osawa 2011) note that the cannibalistic interactions within species may be strong enough to lessen the negative impact of intraguild predation of *H. axyridis* on *A. bipunctata*. Furthermore, Van Rijn et al. (2005) argue intraguild predation may be strong enough for the exotic species to invade a stable predator-prey system but that the invasive species will not displace native species when certain levels of cannibalism exist. Moreover, smaller species that have a size-disadvantage in intraguild predation may have an advantage in resource competition since they can survive and develop on lower prey densities than larger species (Van Rijn et al. 2005).

Numbers of native species have declined in southwestern Michigan, USA, since the arrival of exotic ladybird species, and the diversity of native species has changed when measured on a weekly basis, but it did not change on a yearly basis (Bahlai et al. 2013). Complete elimination of native species is seldom observed in association with the arrival of an exotic ladybird in the USA (Harmon et al. 2007; Bahlai et al. 2013).

Reduction in numbers of several introduced invasive species has been observed after some or many years (Simberloff and Gibbons 2004); besides, some invasive alien

ladybird species seem to decline with time, e.g. *Coccinella undecimpunctata* L. and *C. septempunctata* in the USA (Evans et al. 2011). Hence, the current situation in Europe may not be the terminal stage but a transition to a new balance where native species are strongly reduced in abundance, but do not go extinct.

What next?

In this thesis I show that no single life history characteristic can explain the invasion success of *H. axyridis*, but instead several life history characteristics and their interactions play a role. This is neither surprising nor unexpected, but I propose that future studies should use a multilevel or integrated approach, testing those characteristics simultaneously and in interaction, preferably under field conditions. Emphasis should be placed on experiments in populations in cages where individual factors can be altered and tested in combinations, e.g. densities, age structure, species composition, natural enemy composition, and food availability. Thus, a situation is created that is as close to natural conditions as possible and that allows experiments to be designed that go beyond mere field observations. Field observations will still be needed to provide basic data on, for example, timing, densities, location, etc., and to verify results of controlled field experiments.

Secondly, in order to deepen our understanding of the dynamics of exotic ladybird species it would be worthwhile to compare life histories of several exotic ladybirds that have been introduced but with different levels of invasion success. Why, for example, has the ladybird *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) of which 500 million specimens have been introduced in the Netherlands between 1994 and 2003, not established (Loomans et al. 2013), while *H. axyridis* has? Are there crucial differences in their life history characteristics?

A third suggestion for future research is to study the history and the future role of natural enemies in regulating *H. axyridis* populations. I have found the first infections with different kinds of natural enemies in the Netherlands. Very little is known about these natural enemies in Europe. Future research should focus on the biology, infection mechanisms, and transfer mechanisms of these natural enemies and on the fitness consequences of infection for *H. axyridis* and native ladybird species. This research might result in possibilities for control of *H. axyridis* and reduction of its negative side effects.

Acknowledgements

I would like to thank Joop van Lenteren, Peter de Jong, and Lia Hemerik for their comments on an earlier version of this chapter.

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Summary

The multicoloured Asian ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) was introduced to various areas of the world as a biological control agent of aphids. Since its introduction it has established and spread. Soon after its establishment negative effects on non-target species, fruit production, and human health were reported. *Harmonia axyridis* is now regarded as an invasive species in many parts of the world. Although many characteristics of *H. axyridis* are thought to contribute to its invasion success, a well-documented scientific explanation of this success has been lacking so far. The aim of my thesis research was to determine the causes and consequences of the establishment and rapid spread of *H. axyridis* in the Netherlands.

In **chapters 2 and 3** I address the question whether the mode of overwintering of *H. axyridis* – in diapause and/or in a quiescent state – might be an explanation for its invasion success. The intensity and length of dormancy of *H. axyridis* was determined by collecting hibernating individuals at various locations in the wild and transferring them to outdoor cages to continue overwintering (**chapter 2**). From the end of November the intensity of dormancy was studied at two weekly intervals by recording the pre-oviposition period and ovarian development of individuals that were transferred to long-day, warm conditions in the laboratory. Pre-oviposition periods were short throughout our observations, indicating that *H. axyridis* was not in diapause at the start of our observations but in a quiescent state. Thus, *H. axyridis* can become active rapidly when temperature rises in spring, but, nevertheless, it is not reported to be active earlier in the year than native species.

In **chapter 3** I continued the previous study and tested whether in northwestern Europe *H. axyridis* has a short and early period of real diapause starting at the end of October and shifts to a quiescent state in December. For this study ladybirds were sampled from their hibernation sites immediately after their migratory flights in October. Subsequently, they were kept in outdoor cages, and then, after certain time intervals, the pre-oviposition time was measured under optimal egg-laying laboratory conditions at 25°C at both short (D:L = 12:12) and long (D:L = 8:16) photoperiods. During the first part of the experiment I observed a significant, albeit small, difference in pre-oviposition period of ladybirds reared at the two photoperiods. This difference disappeared in December, indicating that ladybirds are in a state of diapause until mid-December, and from then on in a quiescent state, as I found in **chapter 2**. The findings indicate that the diapause of *H. axyridis* generally is relatively short and weak compared with published data of native ladybirds. Moreover, it appears to have become shorter over the last decade.

Overwintering survival of five wild *H. axyridis* populations in the Netherlands under natural and semi-natural conditions was described in **chapter 4.** A high overwintering survival results in a large post-hibernation population and a rapid population build-up in spring. I focussed on the potential influence on survival of location and orientation of ladybird aggregations in overwintering sites. In the Netherlands, overwintering survival of *H. axyridis* is high: 70.8 to 88.2%. At all five sample sites, ladybirds that were hibernating at the southwestern sides of buildings, where most aggregations of ladybirds were found, had a higher winter survival than ladybirds hibernating at other orientations. At sheltered sites survival was higher compared to exposed sites. Compared with most common native species, winter

survival of *H. axyridis* is similar or higher.

Many earlier studies identifying life history characteristics were performed under laboratory conditions, impairing extrapolation to field conditions. Therefore, in my study I looked at life history characteristics of *H. axyridis* and *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae) under semi-field conditions. This part of my research is described in **chapter 5**. Larvae were placed on lime trees in single- or mixed-species groups and provided with ample food. Immature development time and survival were estimated for both species. Development time was in line with data from laboratory experiments under controlled conditions for both species. Immature survival reached high levels (i.e. 44.4–100% for *H. axyridis* and 11.1–76.9% for *A. bipunctata*), but survival was generally considerably higher for *H. axyridis* than for the native *A. bipunctata*. I did not observe an effect of the heterospecific groups on the development of *A. bipunctata* and *H. axyridis* when sufficient prey was available, and intraguild predation did not seem to cause important mortality at low larval densities and conditions of high prey availability.

Intraguild predation behaviour in absence of food was studied under field conditions for three ladybird species: *Coccinella septempunctata* L. (Coleoptera: Coccinellidae), *A. bipunctata,* and *H. axyridis*) (**chapter 6**). Predation behaviour of fourth instar larvae of these three species was investigated in semi-field experiments on small lime trees. During the 3-hour observations, the two fourth instar larvae placed on a tree rarely made contact. When placed together on a single leaf at least one contact was made in 23–43% of the observations, depending on the tested species combination. At the most 27% of those contacts resulted in an attack. *Adalia bipunctata* and *C. septempunctata* attacked heterospecifics as often as conspecifics, while *H. axyridis* attacked mostly heterospecific larvae. *Harmonia axyridis* won 86% and 44% of heterospecific battles against *A. bipunctata* and *C. septempunctata* respectively. *Coccinella septempunctata* won only the heterospecific battles against *A. bipunctata*, and *A. bipunctata* did not win any of the heterospecific battles. The results of these semi-field experiments confirm that *H. axyridis* is a strong intraguild predator; this is probably a consequence of its aggressiveness and good defence against predation from heterospecific species.

Knowledge of natural enemies of *H. axyridis* is summarised in **chapter 7**. During the past ten years ladybirds were sampled from hibernation aggregations in winter and from spring through to autumn with illuminated screens at night by PS van Wielink. Additionally I have collected beetles for my experiments In winter 2008, 2009, and 2010. From those samples ladybirds were checked for presence of natural enemies. In the samples from 2003—2007 no natural enemies were found. From 2008 onwards *H. axyridis* adults were infested by *Hesperomyces virescens* Thaxt. fungi (summer and winter), *Parasitylenchus bifurcatus* Poinar and Steenberg nematodes (winter), *Coccipolipus hippodamiae* (McDaniel and Morrill) mites (winter), and *Dinocampus coccinellae* (Schrank) parasitoids (summer and winter). Our results indicate that these natural enemies are starting to use *H. axyridis* as a host but are as yet not sufficiently abundant and/or effective to have a profound impact on populations of the invader.

In **chapter 8**, I provide an overview of several life history traits of *H. axyridis*. I first investigated whether invasive populations differ from the native Asian populations of

H. axyridis. I performed a meta-analysis to determine the effect of geographic origin, photoperiod, food, ladybird strain, and temperature on development and survival of the egg, larval, and pupal stages and on four post-eclosion parameters (pre-oviposition period, generation time, reproduction, and longevity) of *H. axyridis*. Differences in several life history characteristics were found between Asian and invasive populations of *H. axyridis,* which suggests that the invasive populations developed from a non-random subset of the beetles in the area of origin, or that evolutionary changes have occurred after *H. axyridis* invaded new areas. The greatest differences in development rate were observed at temperatures above 24°C while at temperatures ranging from 17 to 24°C, individuals of invasive populations and native Asian populations develop at similar rates.

Next, I compared life cycle parameters of invasive *H. axyridis* and native European ladybirds *A. bipunctata, C. septempunctata,* and *Propylea quatuordecimpunctata* (L.) (Coleoptera: Coccinellidae). Compared to native species, *H. axyridis* develops slower and starts reproduction later, suggesting no competitive advantage for the invader. However, it has a higher longevity, fecundity, and number of generations per year, and as a result *H. axyridis* can potentially easily outnumber native species within a few years. In addition, this species is the winner in most intraguild predation combinations with native ladybirds.

In **chapter 9** I summarise and discuss the work described in this thesis. At temperatures characteristic for spring and summer in northwestern Europe (17 to 24°C), invasive populations of *H. axyridis* do not differ from native Asian populations; thus, the invasion success cannot be attributed to a change in life history characteristics of the invasive population. Compared with native ladybird species, the exotic species lives longer, lays more eggs, and has more generations per year, which is expected to result in a faster population growth of *H. axyridis*.

Although under semi-field conditions with high food availability, no effect of the presence of *H. axyridis* on the survival, development, weight, and size of the native species *A. bipunctata* was found, intraguild predation may occur under natural conditions as aphid colonies are relatively short-lived which causes prey scarcity. When contacts between ladybirds do occur, *H. axyridis* larvae are the winners in contacts with larvae of the native species *A. bipunctata* and *C. septempunctata*. Being a strong intraguild predator, *H. axyridis* compensates its slow immature development and late arrival at aphid colonies compared to native species its ability to feed on eggs, larvae, and pupae of other ladybirds, thereby completing its development. The successful invasion of *H. axyridis* in the Netherlands can be explained by the combination of characteristics, mentioned above, namely overwintering, immature survival, fecundity, longevity, number of generations per year, and intraguild predation.

Several facts such as 1) the quite stable diversity and abundance of ladybird species in Asia, 2) the observed reduction in abundance of several introduced invasive plant and animal species after some time, 3) the observation that populations of some invasive alien ladybird species decline with time; and 4) the first evidence that natural enemies start to use *H. axyridis* as host in the invaded range, suggest that the current situation in Europe may not be the terminal stage but a transition to a new balance where native species are strongly reduced in abundance, but do not become extinct.

Samenvatting

Het veelkleurige Aziatische lieveheersbeestje *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) is in het verleden in verschillende delen van de wereld geïntroduceerd als biologische bestrijder van bladluizen. Na introductie heeft het lieveheersbeestje zich gevestigd en verspreid. Kort nadat het zich had gevestigd, werden negatieve effecten gemeld op fruitproductie, menselijke gezondheid en op insectensoorten waartegen het lieveheersbeestje niet doelbewust was ingezet. Tegenwoordig wordt *H. axyridis* in veel delen van de wereld beschouwd als een invasieve soort. Hoewel *H. axyridis* veel kenmerken heeft waarvan wordt aangenomen dat deze bijdragen aan diens invasieve succes, heeft het tot nu toe aan een goed gedocumenteerde wetenschappelijke verklaring voor dit succes ontbroken. Het doel van mijn promotieonderzoek was om de oorzaken en gevolgen van de vestiging en snelle verspreiding van *H. axyridis* in Nederland te bepalen.

In **hoofdstuk 2 en 3** ga ik in op de vraag of de wijze van overwinteren van *H. axyridis* – diapauze of winterrust – een verklaring zou kunnen vormen voor zijn invasieve succes. De intensiteit en duur van de overwintering van *H. axyridis* werden bepaald door op verschillende plaatsen in het wild overwinterende individuen te verzamelen en te verplaatsen naar kooien in de buitenlucht om daar verder te overwinteren (**hoofdstuk 2**). Vanaf eind november werd iedere twee weken de intensiteit van de overwintering gemeten door de tijd tot aan het leggen van het eerste ei (= pre-ovipositie periode) en de ontwikkeling van de ovaria van individuele kevers te bepalen, nadat deze waren overgebracht naar warme omstandigheden in het laboratorium bij 16 uur licht. Wij namen alleen korte pre-ovipositie perioden waar, wat erop wijst dat *H. axyridis* aan het begin van onze waarnemingen niet in diapauze maar in winterrust verkeerde. Bij stijgende temperaturen in de lente kan *H. axyridis* daardoor snel actief worden; desondanks wordt in het wild niet waargenomen dat *H. axyridis* in het voorjaar eerder actief is dan inheemse soorten.

In **hoofdstuk 3** ging ik verder met het hierboven genoemde onderzoek en onderzocht ik of *H. axyridis* in Noordwest-Europa vroeger eerst een vroege en korte periode heeft waarin het echt in diapauze verkeert. Deze periode zou dan eind oktober beginnen en in december overgaan in winterrust. Voor dit onderzoek werden steekproeven genomen op de overwinteringsplekken, meteen na de migratievluchten in oktober. De lieveheersbeestjes werden vervolgens in kooien in de buitenlucht gehouden en na bepaalde tijdsintervallen werd de tijd tot aan pre-ovipositie gemeten onder laboratoriumomstandigheden die optimaal waren voor het leggen van eieren: 25°C bij zowel korte (D:L = 12:12) als lange (D:L = 8:16) dag. Tijdens het eerste deel van het experiment vond ik een significant maar klein verschil in pre-ovipositie periode van de lieveheersbeestjes die bij de twee verschillende daglengten gehouden waren. Dit verschil verdween in december, wat erop wijst dat lieveheersbeestjes tot half-december in diapauze verkeren en daarna overgaan tot winterrust. Dit komt overeen met wat ik in **hoofdstuk 2** heb gevonden. De bevindingen geven aan dat de diapauze van *H. axyridis* in het algemeen relatief kort en zwak is vergeleken met gepubliceerde gegevens over inheemse lieveheersbeestjes. Bovendien lijkt het erop dat de diapauze de laatste tien jaar korter is geworden.

De wintersterfte van vijf wilde populaties van *H. axyridis* is onderzocht onder natuurlijke en semi-natuurlijke omstandigheden. Deze studie is beschreven in

hoofdstuk 4. Als een groot aandeel van de populatie de winter overleeft, leidt dat tot een grote startpopulatie in het voorjaar en vervolgens een snelle populatieopbouw. Ik richtte mij op de mogelijke invloed van de positionering en oriëntatie van de groepen overwinterende *H. axyridis* op de wintermortaliteit van de kevers in de groepen. Op beschutte plekken was de overleving hoger dan op onbeschutte plekken. Vergeleken met de meeste algemeen voorkomende inheemse soorten is winteroverleving van *H. axyridis* vergelijkbaar of hoger.

Veel eerdere onderzoeken naar de eigenschappen van *H. axyridis* werden in het laboratorium uitgevoerd, waardoor de resultaten niet geëxtrapoleerd konden worden naar veldomstandigheden. Daarom heb ik de eigenschappen van *H. axyridis* en *A. bipunctata* (L.) (Coleoptera: Coccinellidae) onder semi-natuurlijke omstandigheden bekeken. Dit deel van mijn onderzoek is beschreven in **hoofdstuk 5**. Larven die net uit het ei waren gekropen, werden in homogene of heterogene groepen op lindes (*Tilia platyphyllos*) gezet waarop ook ruim voldoende bladluizen als voedsel aanwezig waren. De ontwikkelingstijd en overleving tot het uit de pop kruipen werden geschat. Voor beide soorten kwam de ontwikkelingstijd overeen met gegevens uit laboratorium-experimenten onder gecontroleerde omstandigheden. De overleving van larven en poppen was voor beide soorten hoog (44.4—100% voor *H. axyridis* en 11.1—76.9% voor *A. bipunctata*), maar in het algemeen was de overleving voor *H. axyridis* aanzienlijk hoger dan voor de inheemse soort *A. bipunctata*. Onder de geteste omstandigheden, bij beschikbaarheid van voldoende voedsel en lage larven-dichtheden, hebben we geen effect gevonden van de heterogene groepen op de ontwikkelingsduur van *A. bipunctata* en *H. axyridis* en onderlinge predatie leek geen belangrijke oorzaak te zijn van sterfte tijdens de ontwikkeling.

Predatie tussen lieveheersbeestjes onderling bij een voedseltekort onder semi-natuurlijke omstandigheden werd onderzocht voor drie soorten lieveheersbeestjes: *Coccinella septempunctata* L. (Coleoptera: Coccinellidae), *A. bipunctata* en *H. axyridis* (**hoofdstuk 6**). Het predatiegedrag van vierde-stadium larven van deze drie soorten werd geobserveerd op kleine lindes. Gedurende de 3 uur durende observaties was er zelden contact tussen de twee larven op een boom. Als de larven samen op één blad gezet werden, werd er in 23–43% van de gevallen minstens één keer contact gemaakt, afhankelijk van de onderzochte soortencombinatie. Hooguit 27% van deze ontmoetingen leidde tot een aanval. *Adalia bipunctata* en *C. septempunctata* vielen even vaak larven van de eigen soort aan als larven van een andere soort, terwijl *H. axyridis* vooral larven van de andere soort aanviel. Van de gevechten tussen twee soorten won *H. axyridis* respectievelijk 88% en 44% van de gevechten met *A. bipunctata* en *C. septempunctata*. In de andere gevallen was er geen winnaar. *Coccinella septempunctata* won alleen de gevechten met *A. bipunctata* en *A. bipunctata* won geen enkel gevecht met een andere soort. De resultaten van deze semi-veldexperimenten bevestigen dat *H. axyridis* een sterke predator is van andere soorten; dit is waarschijnlijk een gevolg van zijn agressiviteit en van zijn goede afweer tegen predatie door andere soorten.

Kennis over natuurlijke vijanden van *H. axyridis* is samengevat in **hoofdstuk 7**. De afgelopen tien jaar zijn door PS van Wielink lieveheersbeestjes verzameld: in de winter uit overwinterende groepen en in de lente tot aan de herfst werden ’s nachts vliegende lieveheersbeestjes gevangen met verlichte schermen. Daarnaast heb ik in de

winters van 2008, 2009 en 2010 lieveheersbeestjes verzameld voor mijn experimenten. De verzamelde lieveheersbeestjes werden onderzocht op de aanwezigheid van natuurlijke vijanden. In de steekproeven van 2003—2007 werden deze niet gevonden. Vanaf 2008 waren volwassen exemplaren van *H. axyridis* 's zomers en 's winters aangetast door schimmels (*Hesperomyces virescens* Thaxt.) en parasitoïden (*Dinocampus coccinellae* (Schrank)) en 's winters bovendien door nematoden (*Parasitylenchus bifurcatus* Poinar en Steenberg) en mijten (*Coccipolipus hippodamiae* (McDaniel en Morrill)). Onze resultaten geven aan dat deze natuurlijke vijanden *H. axyridis* als gastheer beginnen te gebruiken, maar dat ze tot nu toe nog niet voldoende wijdverspreid en/of effectief zijn om een verregaande invloed te hebben op de populaties van de indringer.

In **hoofdstuk 8** geef ik een overzicht van een aantal kenmerken van *H. axyridis*. Ik heb eerst onderzocht of invasieve populaties van *H. axyridis* verschillen van de inheemse Aziatische populaties. Daarvoor heb ik een meta-analyse uitgevoerd om het effect te bepalen van geografische oorsprong, fotoperiode, voedsel, lieveheersbeestjes stam (wild of gekweekt) en temperatuur op de ontwikkeling en overleving van het ei stadium, de larvale stadia, het pop stadium en op vier parameters van de volwassen kever (pre-ovipositie periode, generatieduur, het aantal gelegde eitjes en levensduur van *H. axyridis*). Voor een aantal kenmerken heb ik verschillen gevonden tussen de Aziatische en invasieve populaties, wat aan lijkt te geven dat de invasieve populaties zijn voortgekomen uit een bepaalde subgroep van kevers in het gebied van oorsprong, of dat er evolutionaire veranderingen hebben plaatsgevonden nadat *H. axyridis* zich in nieuwe gebieden had gevestigd. De grootste verschillen in ontwikkelingssnelheid werden waargenomen bij temperaturen boven 24°C, terwijl bij temperaturen van 17 tot 24°C individuen van invasieve en inheemse Aziatische populaties zich met gelijke snelheid ontwikkelen.

Vervolgens vergeleek ik levensloopparameters van invasieve *H. axyridis* en de inheemse Europese lieveheersbeestjes *A. bipunctata*, *C. septempunctata*, en *Propylea quatuordecimpunctata* (L.) (Coleoptera: Coccinellidae). Vergeleken met inheemse soorten ontwikkelt *H. axyridis* zich langzamer en begint later met de voortplanting, wat erop lijkt te duiden dat deze indringer in dit opzicht geen (concurrentie)voordeel heeft. Echter, *H. axyridis* heeft een langere levensduur, legt meer eieren en brengt per jaar meer generaties voort, waardoor *H. axyridis* gemakkelijk binnen een paar jaar inheemse soorten in aantal kan overvleugelen. Bovendien komt deze soort als winnaar uit de strijd in gevechten met de meeste andere soorten lieveheersbeestjes.

In **hoofdstuk 9** vat ik het werk dat in dit proefschrift is beschreven samen en geef ik een interpretatie van de resultaten. Bij temperaturen die in de lente en zomer in Noordwest-Europa gebruikelijk zijn (17 tot 24°C), verschillen invasieve *H. axyridis*-populaties niet van inheemse Aziatische populaties. Het invasiesucces kan dus niet worden toegeschreven aan een verandering in kenmerken van de invasieve populatie. In vergelijking met inheemse lieveheersbeestjes soortenleeft de exotische soort langer, legt hij meer eieren en brengt hij per jaar meer generaties voort. De verwachting is dat dit resulteert in een snellere populatiegroei van *H. axyridis*.

Hoewel er onder semi-veldomstandigheden met voldoende voedsel geen effect gevonden werd van de aanwezigheid van *H. axyridis* op de overlevingskansen,

ontwikkeling, gewicht en grootte van de inheemse soort *A. bipunctata*, kan predatie tussen soorten toch voorkomen onder natuurlijke omstandigheden aangezien bladluizenkolonies relatief kort bestaan wat uiteindelijk prooischaarste veroorzaakt. Als larven van lieveheersbeestjes elkaar ontmoeten, winnen *H. axyridis*-larven gevechten met larven van de inheemse soorten *A. bipunctata* en *C. septempunctata*. Doordat *H. axyridis* een sterke predator is van larven van zowel andere soorten als van de eigen soort, worden zijn langzame ontwikkeling en – vergeleken met inheemse soorten – late aankomst bij bladluizenkolonies gecompenseerd door zijn vermogen om van eieren, larven en poppen van andere lieveheersbeestjes te leven en op die manier zijn ontwikkeling te voltooien. De succesvolle invasie van *H. axyridis* in Nederland kan worden verklaard door de combinatie van kenmerken die hierboven zijn genoemd: overleving in de winter, overleving van larven en pop, levensduur, reproductie, aantal generaties per jaar en predatie van andere lieveheersbeestjes van de eigen soort en van andere soorten.

Verschillende feiten, zoals 1) de vrij stabiele diversiteit en wijdverspreide aanwezigheid van soorten lieveheersbeestjes in Azië; 2) de waarneming dat de populatiedichtheid van geïntroduceerde invasieve planten en dieren op den duur afneemt; 3) de waargenomen vermindering in aantallen van sommige invasieve exotische lieveheersbeestjessoorten in de loop van de tijd; en 4) het eerste bewijs dat in de gebieden waar *H. axyridis* zich heeft gevestigd, natuurlijke vijanden *H. axyridis* als gastheer beginnen te gebruiken, lijken erop te wijzen dat de huidige situatie in Europa misschien niet het eindstadium is, maar een overgang naar een nieuwe situatie waarin inheemse soorten weliswaar sterk in aantal afgenomen zijn, maar toch niet uitsterven.

Curriculum vitae

Lidwien Raak-van den Berg was born on 4 January 1976 in Breda, the Netherlands. In 1994, after obtaining her VWO degree at the Christelijk Lyceum in Alphen aan den Rijn, she started her study 'Plant breeding and crop protection' at Wageningen Agricultural University. Together with 5 fellow students she was responsible for the organisation of the Annual Introduction Days for the new students of Wageningen University in 1998. As part of her Master's studies she studied the behaviour and development of the Diamondback moth (*Plutella xylostella*) on *Arabidopsis thaliana* wax mutants with in vitro and in planta experiments. During her internship she conducted fieldwork in Costa Rica to study densities and spatial distribution of whiteflies in the natural environment of its natural enemy *Encarsia formosa*. Afterwards she extended her curriculum with courses in the field of communication and statistics.

After obtaining her Master's degree (with distinction) in 2002, Lidwien worked at the Plant Protection Service on a project that aimed to make fytosanitary regulations of non-EU countries accessible on the internet. From 2004 onwards she developed databases supporting plant breeding for breeding companies as a business consultant of AgriInformationPartners. In 2008 she started her PhD-project at the Laboratory of Entomology of Wageningen University, under the supervision of Joop C van Lenteren, Peter W de Jong, and Lia Hemerik. The research focussed on identifying what life history characteristics might explain the invasion success of *Harmonia axyridis* in the Netherlands. Lidwien has either published or submitted all experimental chapters of her thesis and presented her work at several national and international meetings. In addition she supervised and trained four BSc-students and two MSc-students.

After defending her PhD thesis Lidwien will continue her career as Officer Plant Health at the Netherlands Food and Consumer Product Safety Authority (NVWA).

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Life table of *Harmonia axyridis* and *Adalia bipunctata* under semi-field conditions

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Dankwoord

Heel blij ben ik met mijn proefschrift dat nu voor u ligt. Na een mooie, leerzame en intensieve periode is het goed om het af te ronden. Ik heb er hard en veel aan gewerkt, maar dit proefschrift had niet tot stand kunnen komen zonder de steun, hulp en bijdrage van velen. Het is moeilijk om volledig te zijn, maar hieronder zal ik een poging doen om mensen te bedanken.

Laat ik met het thuisfront beginnen. Levende beestjes houden zich meestal niet aan werkdagen en weekenden, dus zonder begrip thuis en zo nu en dan een helpende hand, zou het erg moeilijk zijn geweest de proeven uit te voeren. Lieve Michiel, dank voor je uithoudingsvermogen, geduld en alle extra inspanningen om het thuis allemaal draaiende te houden. Zeker met een kleintje dat ritme en regelmaat nodig heeft, is dat niet altijd gemakkelijk. Je had een luisterend oor bij frustraties en successen en je frisse blik hielp om dingen vanuit een ander gezichtspunt te bekijken. Dank voor je begrip voor alle uren die ik op vreemde tijdstippen in mijn onderzoek moest steken. Fijn dat je ook hebt geholpen met de kweek en de waarnemingen.

Hidde, mijn kleine schat, jij zorgde ervoor dat ik, zodra ik thuis kwam, zeker niet met mijn werk bezig kon zijn. Een hele goede afwisseling. Gelukkig was je een liefhebber van lange middagslaapjes en heb ik op mama-dagen regelmatig nog een uurtje kunnen werken. Tenzij ik erop rekende, natuurlijk. Imke, mijn kleine meisje, je zal wel wat drukte gevoeld hebben toen je in mijn buik groeide, want er moest nog veel gebeuren voordat de hoofdstukken inhoudelijk allemaal goed waren. Maar het is gelukt en ik heb zelfs nog even van de rust kunnen genieten voordat je jezelf aankondigde.

Mama en papa, bedankt voor jullie steun en de aanmoediging om aan dit promotietraject te beginnen. Jullie hebben me altijd gestimuleerd om het beste uit mezelf te halen. Zonder jullie had ik dit niet bereikt.

Veel hulp heb ik ook gehad van oma Marianne, die één dag per week de zorg voor Hidde op zich nam en vele hand- en spandiensten heeft verricht. Opa Lode bedankt voor het klussen in huis, zodat ik daar zelf geen tijd in hoefde te stoppen. Saskia, ik vond het heel fijn dat je steeds de tijd hebt willen nemen om mijn manuscripten te lezen en te voorzien van commentaar; daar heb ik veel aan gehad! Oom Carl, Oom Ed en Tineke, dank voor jullie hulp en ondersteuning. Jullie hadden altijd een belangstellend oor voor de vorderingen en waren enthousiast verzamelaars van aan mijn onderzoeksonderwerp gerelateerde krantenknipsels.

Zonder inhoudelijke begeleiding van mijn (co-)promotoren had dit boekje nu niet voor u gelegen. Joop, dank voor je uitgebreide en kritische correcties en commentaren, in combinatie met je snelle respons, waar ter wereld je je ook bevond (dat kon ik niet altijd bijhouden). Hierdoor hield je me op het juiste spoor en kon ik verder. Ik heb veel van je geleerd. Peter, bij jou kon ik binnenlopen voor praktische zaken tijdens de experimenten, voor vragen over analyses, interpretaties en in de schrijffase. Dank voor de tijd en energie die je aan me hebt besteed. Lia, jij werd van survival-analyse-docent, een co-auteur en uiteindelijk co-promotor; een rol de je met verve op je genomen hebt. Dank je voor je support bij het eindeloze analyseren. En niet te vergeten voor je hulp bij de creatie van de figuren in R.

Mijn co-auteurs (Gerrit Gort, Danny Haelewaters, Hans Helder, Marieke de Lange, Bryan Manly, Jeltje Stam, Wopke van der Werf en Paul van Wielink) ben ik ook dank

verschuldigd voor hun diverse bijdrages op hun eigen vakgebied. Marieke en ik hebben heel wat tropische temperaturen getrotseerd. Marieke, het was een prettige samenwerking en ik vond het leuk dat we echt samen alles gedaan hebben van proefopzet tot indienen. Ik heb veel van je geleerd en hoop de samenwerking voort te zetten. Toen Jeltje haar afstudeeronderzoek deed, hebben we juist in de kou gewerkt. Uren hebben we met muts, wanten, jas, kruik en dekens doorgebracht in de koelcel, terwijl we keken naar de wintermortaliteit van *H. axyridis.* Als ik geen hulp had gehad van de studenten Anouk, Ellard, Ilona, Jos en Walinka, dan had mijn proefschrift een stuk minder data bevat. Dank voor jullie harde werken.

Zonder beestjes geen experimenten. Veel mensen hebben gereageerd op mijn oproep om contact op te nemen als ze lieveheersbeestjes in huis hadden. Bij velen mocht ik langs komen en kevers verzamelen, waarvoor ik dankbaar ben. Op een aantal plaatsen heb ik ze meerdere keren mogen verzamelen en mocht ik zelfs overwinteringskooitjes plaatsen. Hartelijk dank voor de hulp en de gastvrijheid: familie Brandsma, K Helling (gemeente Houten), familie Huijsman, familie Van der Kamp, familie Schep en E Voorhout. Antoon, bedankt dat je me vergezeld hebt op verzameltochtjes en voor de tips en praktijkervaring die je hebt willen delen. En als je de beestjes eenmaal hebt, moeten ze ook nog eten: Koppert en Entocare bedankt voor de tienduizenden luizen en *Ephestia* eieren..

Andries Siepel, Gerard Mekking en Henk van Roekel hebben me elke keer weer met praktische raad en daad en de nodige mankracht terzijde gestaan tijdens mijn veldexperimenten en de voorbereiding ervan. Yde heeft me geholpen met de eerste experimenten in de winter.

Jetske en Karen, wat fijn dat jullie mijn paranimf willen zijn. Jetske, ik ken je onderhand al meer dan de helft van mijn leven. We hebben heel wat zaken (werkgerelateerd of niet) besproken tijdens onze lunchwandelingen en jouw heldere kijk op de zaken helpt me. Karen, met jou was het altijd erg gezellig pauzeren en jij hebt me in de laatste fase veel geholpen, onder andere met figuren in R.

I loved my time at the laboratory for Entomology and enjoyed the company of all colleagues during coffee and lunch breaks, but also during parties, drinks and “lab-uitjes”. The PhD-lunches and other discussions with fellow PhD’s were inspiring and encouraging, thank you for that. I also thank those that were willing to share materials, knowledge, and time in the weekends by watering the plants or checking that last petri-dish, allowing me to leave for the weekend. I was very happy to be able to benefit from the intenational character of Entomology: several colleagues helped me with the translation of manuscripts – Keiko, Yutong, Shuhang, Si-jun, Penjung, Midori, Dani, and Camille – or read the manuscript when reviewers required it to be checked by a native speaker – Alex. Thanks to you all.

Het laatste jaar ben ik gastvrij geadopteerd door Biometris, waardoor ik een werkplek had om mijn proefschrift af te ronden. Dank hiervoor. Ik vond het leuk jullie te leren kennen en heb veel geleerd. Dinie, je hebt de vormgeving van dit proefschrift gedaan; bedankt voor jouw inspanningen en geduld. Het waren heel veel puntjes op heel veel i’s. Bas met jou heb ik ook heel wat inhoudelijke discussies gevoerd. We deden veldwerk op dezelfde locatie en het laatste jaar was je een prettige

sparringpartner voor teksten en analyses. Mireille en Katja, de eerste tijd nadat Hidde geboren was, hebben we samen bijna dagelijks het nuttige en aangename verenigd.

Niet alleen het thuisfront, collega's en vakgenoten hebben mij gesteund en geholpen, in mijn vrije uren heb ik ook morele steun en afleiding gehad van vrienden. De lunches op de F. Bordewijk straat waren een rustpunt om stoom af te blazen, bij te komen en zaken vanuit een ander gezichtspunt te bekijken. Susan, Ellen, Jorit en Emmy, ik hoop dat we dit kunnen blijven doen. Voor ontspanning tijdens een wandeling, muziek op schoot, sporten of gewoon voor een kopje thee of een avondje in de kroeg kon ik terecht bij Ilse, Jessica, Esther, Marion, Welmoed en Ghyslaine. Bij de buren (Dick, Ineke, Jantiene en Enide) voor een praatje of opvang van zoonlief. Ireen, Lidwine, Marjan, Marielle, Zwannet, Mijke en Cecile, ondanks dat we allemaal opgeslokt worden door werk en gezin, ben ik blij dat we steeds een luisterend oor vinden bij elkaar. Saskia H, als je kwam logeren voerden we veel interessante gesprekken over belangrijke zaken in het leven.

Allemaal − hopelijk ben ik niet teveel mensen vergeten − bedankt dat jullie er waren.

Lidwien

Education statement

PE&RC Training and Education Statement

With the training and education activities listed below, the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- Did the life of multicoloured Asian ladybird *Harmonia axyridis* change when it spread across the globe?- a meta-analysis

Post-graduate courses (5.8 ECTS)

- Biodiversity and ecosystem services in a sustainable world; PE&RC (2008)
- Life history theory; FE, Schiermonnikoog (2009)
- Survival analysis; PE&RC (2009)
- Monitoring and resource sampling; Alterra (2009)

Laboratory training and working visits (0.9 ECTS)

- Sampling and recognizing *Harmonia axyridis* and other ladybirds; Plantenziektekundige dienst, Wageningen (2008)

Invited review of (unpublished) journal manuscript (2 ECTS)

- Entomologia Experimentalis et Applicata: *Eriopis connexa* and *Hippodamia variegata* in Chile (2012)
- Insect Conservation and Diversity: escape from parasitism by *Harmonia axyridis* (2013)

Deficiency, refresh, brush-up courses (1.5 ECTS)

- Basic statistics (2008)

Competence strengthening / skills courses (4 ECTS)

- PhD Competence assessment; WGS (2009)
- Scientific writing; WGS (2010)
- Techniques for writing and presenting a scientific paper; WGS (2011)
- Effective behaviour in your professional surroundings; WGS (2011)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.1 ECTS)

- PE&RC PhD Weekend (2008)
- PE&RC Seminar (2010, 2012)
- PE&RC Last years weekend (2013)

Discussion groups / local seminars / other scientific meetings (7.5 ECTS)

- Jaarvergadering Koninklijke Nederlandse Plantenziektekudige vereniging (2008)
- Entomology PhD lunch discussion group (2008-2013)
- Insect Plant Interactions discussion group (2008-2013)
- Netherlands Annual Ecology Meeting (2009, 2010, 2011, 2013, 2014)
- Nederlandse Entomologische Vereniging - Entomologendag (2009, 2010, 2012, 2013)

International symposia, workshops and conferences (7.3 ECTS)

- Working group “Benefits and risks of exotic biological control agents” 1; poster presentation; Engelberg, Switzerland (2009)
- Ecology of Aphidophaga 11; oral and poster presentation; Perugia, Italy (2010)
- Neobiota 7; oral presentation; Pontevedra, Spain (2012)

Lecturing / supervision of practical’s / tutorials (3 ECTS)

- Biology & management of plant pathogens, pests and diseases 2 (2008)
- Molecular & evolutionairy ecology (2009, 2010)
- Biology & management of plant pathogens, pests and diseases 1 (2009, 2010, 2011)
- Bsc Students (4) supervision (2010-2011)
- Behavioural ecology (2012)

Supervision of MSc students (2 ECTS)

- The spread, abundance and life history of *Harmonia axyridis* in the Netherlands
- Overwintering strategy of *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) may contribute to high invasiveness

Colophon

Cover design	Rosemarie van Boxel, KROOS tekst en ontwerp, Soest
Cover photgraph	Peter van Wieringen natuurfotografie, www.natuurenfoto.nl
Photographs	Nina Fatouros, www.bugsinthepicture.com (pages 6, 126, 172, and 209) André de Kesel (page 102) Marieke de Lange (pages 84 and 101) Lidwien Raak-van den Berg (pages 14, 64, and 81) Jeltje Stam (pages 32 and 46)
Layout	Dinie Verbeek, Biometris Lidwien Raak-van den Berg
Printed by	GVO drukkers & vormgevers BV