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Working group "Benefits and Risks of Exotic Biological Control Agents"

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"*Harmonia axyridis* and other ladybirds"

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Dirk Babendreier, Alexandre Aebi, Marc Kenis & Helen Roy

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The Publication Commission of the IOBC/WPRS:

Dr. Ute Koch
Schillerstrasse 13
D-69509 Moerlenbach (Germany)
Tel +49-6209-1079, Fax +49-6209-4849
e-mail: u.koch_moerlenbach@t-online.de

Dr. Horst Bathon
Julius Kühn-Institute (JKI)
Federal Research Center for Cultivated Plants
Institute for Biological Control
Heinrichstr. 243
D-64287 Darmstadt (Germany)
Tel +49 6151 407-224, Fax +49 6151 407-290
e-mail: horst.bathon@jki.bund.de

Address General Secretariat:

Dr. Philippe C. Nicot
INRA – Unité de Pathologie Végétale
Domaine St Maurice - B.P. 94
F-84143 Montfavet Cedex (France)

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Preface

The use of exotic species as biological control agents has a long history and there are many examples of success. Indeed, it was a ladybird (the Vedalia beetle, *Rodolia cardinalis*, introduced from Australia to California to control cottony cushion scale, *Icerya purchasi*, in citrus plantations) that is thought to mark the advent of modern biological control. Classical biological control success stories are undoubtedly numerous and there are very few examples where problems have occurred. *Harmonia axyridis* is, however, one such species. This species was introduced intentionally to a number of countries as a biological control agent of pest insects. Although *H. axyridis* is a success in terms of contributing to the reduction of pest numbers to below economic thresholds, it is considered both a human nuisance in the autumn, as it occupies premises in high numbers, and also a threat to native biodiversity through competition and predation.

This species has spread rapidly across northern and central Europe and is now found at high abundance in many countries. *Harmonia axyridis* has now been recorded as established in (order relates to approximate time of establishment): France (first report), Germany, Belgium, Netherlands, Switzerland, Luxembourg, England, Czech Republic, Italy, Austria, Denmark, Norway, Poland, Wales, Liechtenstein, Scotland, Hungary, Slovakia and Bulgaria (last report). Research to quantify the extent of any negative effects is essential, and collaborative effort is necessary to further understanding of this conspicuous invader. In recognition of this, the IOBC/WPRS (International Organization for Biological Control/ Western Palaearctic Regional Section) Study Group "Benefits and Risks of Exotic Biological Control Agents" was established to encourage collaborations on this species and other exotic biological control agents. This study group is not only a forum for those with an interest in biological control but also offers an opportunity to contribute to understanding of invasive species ecology. The movement of species around the globe has been occurring at a staggering rate in recent decades. Many non-native species are considered to be exquisite additions in their new locality but approximately one percent of new arrivals are considered to pose an unacceptable risk to biodiversity and termed "invasive non-native (=alien) species". *Harmonia axyridis* is widely accepted to be an invasive non-native species and as such provides a model system for invasion biologists.

The first dedicated meeting of this Study Group was held in the beautiful location of Engelberg, Switzerland in September 2009. This four day meeting was a unique opportunity for scientists to gather, share their findings, predictions and future directions on this species and other invasive non-native ladybirds. The collaborative spirit of this IOBC/WPRS Study Group ensures an excellent forum for unravelling the dynamics of *Harmonia axyridis* and other exotic biological control agents.

Helen Roy – Convenor IOBC working group
"Benefits and Risks of Exotic Biological Control Agents"

List of participants

Tim Adriaens
INBO
Kliniekstraat 25
B-1070 Brussels, Belgium
E-mail: tim.adriaens@inbo.be

Alexandre Aebi
Agroscope ART
Reckenholzstrasse 191
8046 Zürich, Switzerland
E-mail: alexandre.aebi@art.admin.ch

Olga Ameixa
Inst. Syst. Biol. Ecol.
Na sadkach 7
37005 Ceske Budejovice, Czech Republic
E-mail: olgameixa@portugalmail.pt

Dirk Babendreier
CABI Bioscience
Rue des Grillons 1
2800 Delémont, Switzerland
E-mail: d.babendreier@cabi.org

Nick Berkvens
Ghent University
Coupure Links 653
9000 Ghent, Belgium
E-mail: Nick.Berkvens@UGent.be

Peter Brown
Anglia Ruskin University
34 Longfields,
Ely CB6 3DN, Great Britain
E-mail: petermjbrown@googlemail.com

Joe Burling
Evolutionary Biology Group
Department of Biological Sciences
University of Hull
HU6 7RX, Great Britain
E-mail: j.m.burling@2005.hull.ac.uk

Richard Comont
CEH Wallingford
Benson Lane,
Crowmarsh Gifford OX10 8BB
Great Britain
E-mail: rimo@ceh.ac.uk

Patrick De Clercq
Ghent University
Coupure Links 653
B-9000 Gent, Belgium
E-mail: Patrick.DeClercq@UGent.be

Anthony Dixon
Inst. Syst. Biol. Ecol.
Na sadkach 7
37005 Ceske Budejovice, Czech Republic
E-mail: a.f.dixon@uea.ac.uk

Arnaud Estoup
INRA
Campus International de Baillarguet
34988 Montferrier-sur-Lez, France
E-mail: estoup@supagro.inra.fr

Edward (Ted) W. Evans
Utah State University, USA
Department of Biology, Utah State University
Logan, UT 84322-5305, USA
E-mail: ewevans@biology.usu.edu

Benoit Facon
INRA
Campus International de Baillarguet
34988 Montferrier-sur-Lez, France
E-mail: facon@supagro.inra.fr

Dominique Fleury
Ecole Ingénieurs Changins
Route de Duillier
1260 Nyon, Switzerland
E-mail: Dominique.Fleury@eichangins.ch

Xenia Fois
ISE-CNR
Traversa la Crucca 3, Regione Baldinca
7040 Li Punti, Italy
E-mail: xeniafois@hotmail.it

Bertrand Gauffre
INRA
400 Route des Chappes
6903 Sophia Antipolis, France
E-mail: Bertrand.Gauffre@sophia.inra.fr

Susanne Harding
LIFE, Univ. of Copenhagen
Thorvaldsensvej 40
1871 Frederiksberg C. (Copenhagen),
Denmark
E-mail: suha@life.ku.dk

Louis Hautier
CRA-W
Rue de Liroux, 2
B-5030 Gembloux, Belgium
E-mail: hautier@cra.wallonie.be

Trond Hofsvang
Bioforsk
Høgskoleveien 7
1432 Ås, Norway
E-mail: trond.hofsvang@bioforsk.no

Alois Honek
Crop Research Institute
Drnovská 507
16106 Prague, Czech Republic
E-mail: honek@vurv.cz

William Hutchison
University of Minnesota
196 Oak Hill Dr.
Shoreview, MN 55126, USA
E-mail: hutch002@umn.edu

Brecht Ingels
Ghent University
Coupure Links 653
9000 Gent, BE
E-mail: Brecht.Ingels@Ugent.be

Firouz Kabiri
BIOTOP
Route de Biot - D4
6100 Valbonne, France
E-mail: fkabiri@biotop.fr

Angelos Katsanis
CABI Europe-Switzerland
Rue des Grillons 1
2800 Delémont, Switzerland
E-mail: a.katsanis@cabi.org

Pavel Kindlmann
Inst. Syst. Biol. Ecol. AS
Na sadkach 7
37005 Ceske Budejovice, Czech Republic
E-mail: pavel.kindlmann@centrum.cz

Lori Lawson Handley
University of Hull
Cottingham Road
HU6 7RX Hull, Great Britain
E-mail: L.Lawson-Handley@hull.ac.uk

Eric Lombaert
INRA Sophia-Antipolis
400 route des Chappes
6903 Sophia-Antipolis, France
E-mail: lombaert@sophia.inra.fr

Zdenka Martinkova
Crop Research Institute
Drnovska 507
16106 Prague, Czech Republic
E-mail: martinkova@vurv.cz

Stavros Kampouris
Tei of Kalamata
Asprochoma
24100 Kalamata, Greece
E-mail: stavroskampouris@hotmail.com

Marc Kenis
CABI Europe-Switzerland
Rue des Grillons 1
2800 Delémont, Switzerland
E-mail: m.kenis@cabi.org

Johannette Klapwijk
IBMA
Veilingweg 17
2651BE Berkel en Rodenrijs, The Netherlands
E-mail: koppertreizen@koppert.nl

Andrew Howe
Forest & Landscape, Unv.Cop
Hoersholm Kongevej 11
DK-2970 Hoersholm, Denmark
E-mail: andy@life.ku.dk

Antoon Loomans
Plant Protection Service
Geertjesweg 15
6706 HC Wageningen, The Netherlands
E-mail: a.j.m.loomans@minlnv.nl

Oldrich Nedved
University of South Bohemia
Branisovska 31
CZ37005 Ceske Budejovice, Czech Republic
E-mail: nedved@prf.jcu.cz

Naoya Osawa
Kyoto University
Kitashirakawa Oiwakecyou
606-8502 Kyoto, Japan
E-mail: osawa@kais.kyoto-u.ac.jp

Christelle Péré
CABI Europe-Switzerland
Rue des Grillons 1
2800 Delémont, Switzerland
E-mail: c.pere@cabi.org

C. Lidwien Raak-van den Berg
Wageningen University
Droevendaalsesteeg 1
Wageningen, The Netherlands
E-mail: Lidwien.Raak-vandenberg@wur.nl

Felipe Ramon Portugal
ENFA
2 route de Narbonne - BP
F-31326 Castanet-Tolosan, France
E-mail: felipe.ramonportugal@educagri.fr

Hans Peter Ravn
Forest & Landscape, Unv.Cop
Hoersholm Kongevej 11
DK-2970 Hoersholm, Denmark
E-mail: hpr@life.ku.dk

Emma Rhule
University of Cambridge
Downing Street,
Cambridge CB2 3EH, Great Britain
E-mail: elr29@cam.ac.uk

Eric Riddick
USDA-ARS
59 Lee Road
Stoneville, MS 38776, USA
E-mail: eric.riddick@ars.usda.gov

Helen Roy
CEH
Benson Lane,
Wallingford OX10 8BB, Great Britain
E-mail: Hele@ceh.ac.uk

David Roy
CEH
Benson Lane,
Wallingford OX10 8EF, Great Britain
E-mail: dbr@ceh.ac.uk

Lene Sigsgaard
Univ. Copenhagen, LIFE
Thorvaldsensvej 40
1871 Frederiksberg C, Denmark
E-mail: les@life.ku.dk

John Sloggett
Tussen Beide Markten 45
9712 CC Groningen, The Netherlands
E-mail: johnsloggett@yahoo.co.uk

António Onofre Soares
University of the Azores
Rua da Mãe de Deus, 13-A
9501-801 Ponta Delgada, Portugal
E-mail: onofre@uac.pt

Riaan Stals
SA Ntl Collect Insects, ARC
Private Bag X134, Queenswood
121 Pretoria, South Africa
E-mail: StalsR@arc.agric.za

George Stathas
Tei Kalamatas & Benaki Inst
ΑΝΤΙΚΑΛΑΜΟΣ
24100- Kalamata, Greece
E-mail: georgestathas@hotmail.com

Tove Steenberg
University of Aarhus
Skovbrynet 14
2800 Kgs. Lyngby, Denmark
E-mail: Tove.Steenberg@agrsci.dk

Guido Sterk
BIOBEST BELGIUM NV
Ilse Velden 18
2260 Westerlo, Belgium
E-mail: INFO@BIOBEST.BE

May-Guri Sæthre
Bioforsk
Høgskoleveien 7
1432 Ås, Norway
E-mail: may-guri.saethre@bioforsk.no

Cathleen Thomas
University of Hull
Cottingham Road,
Hull HU6 9UD, Great Britain
E-mail: c.e.thomas@2007.hull.ac.uk

Alison Thomas
Anglia Ruskin University
East Road,
Cambridge CB1 1PT, Great Britain
E-mail: alison.thomas@anglia.ac.uk

Rumen Tomov
University of forestry
Kliment Ohridski 10
1756 Sofia, Bulgaria
E-mail: rtomov@yahoo.com

Dita Ungerova
University of South Bohemia
Branisovska 31
37005 Ceske Budejovice, Czech Republic
E-mail: dita.ungerova@gmail.com

Remy Ware
University of Cambridge
Department of Genetics, Downing Street,
Cambridge CB2 3EH, Great Britain
E-mail: rlw29@cam.ac.uk

Patricia Wells
Rothamsted Research
Harpenden AL5 2JQ, Great Britain
E-mail: trish.wells@bbsrc.ac.uk

Renate Zindel
ART, Zürich
Reckenholzstrasse 191,
8046 Zürich, Switzerland
E-mail: renate.zindel@art.admin.ch

Contents

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Toward a Noah's Ark for native ladybirds in Belgium?

Tim Adriaens[1], Gilles San Martin[2], Louis Hautier[3], Etienne Branquart[4] & Dirk Maes[1]
[1]Research Institute for Nature and Forest (INBO), Kliniekstraat 25, B-1070 Brussels, Belgium; [2]Behavioural Ecology & Conservation Group - Biodiversity Research Centre - Université catholique de Louvain (UCL) - Croix du Sud 4, B-1348 Louvain-la-Neuve, Belgium; [3]Département Lutte biologique et Ressources phylogénétiques - Centre wallon de Recherches agronomiques, Rue de Liroux, 2, B-5030 Gembloux, Belgium; [4]Belgian Biodiversity Platform - Service Public de Wallonie, Département de l'Etude du Milieu Naturel et Agricole (DGARNE), Avenue Marechal Juin, 23, B-5030 Gembloux- Belgium.
Corresponding author: Tim.Adriaens@inbo.be

The first feral field populations of *Harmonia axyridis* Pallas (HA) in Belgium were discovered in 2001 and most probably originated from a laboratory population nearby (Adriaens *et al.*, 2003). This discovery immediately raised concern over the potential impact of this natural enemy introduction. Studies in North America showed that the species was able to rapidly colonise large areas and become the predominant species in aphidophagous guilds (e.g. Tedders & Schaefer, 1994). Moreover, HA was ranked as a high-risk species in an environmental risk assessment of exotic natural enemies used in inundative biological control (van Lenteren *et al.*, 2003, 2008). Adriaens *et al.* (2003) noted that the species was invading a wide range of semi-natural habitats in Belgium and that it could become a potential threat for native ladybird species and other aphid predators. By 2007, HA had indeed invaded all kinds of urban, agricultural and semi-natural habitats in Belgium. It now also occurs in ecoregions that are rich in native coccinellids and other arthropod groups such as butterflies (Adriaens *et al.*, 2008). Meanwhile, it has already spread to 13 European countries (Brown *et al.*, 2008) and the expansion is still ongoing (e.g. Tomov *et al.*, 2009).

The species is a well-known intraguild predator of almost all western European ladybird species in the laboratory (e.g. Ware *et al.*, 2009, Ware & Majerus, 2008, Kenis *et al.* in prep.). Detailed monitoring of ladybird communities in pine, lime and maple in the Brussels urban area in 2003, 2005 and 2008 revealed changes in native ladybird assemblages and detected a significant decline in the abundance of several ladybird species including the native *Adalia bipunctata* L. and *A. decempuncata* L. This decline coincided with an increase of the HA population (Ottart, 2005, San Martin, 2003). Moreover, analysis of exogenous alkaloid content of HA larvae, sampled on Brussels trees, revealed high levels of intraguild predation (IGP) on native coccinellids, mainly *Adalia* spp., and, to a lesser extent, *Calvia* spp. as well as *Propylea quatuordecimpunctata* L. (Hautier *et al.*, 2008, Hautier *et al.*, 2010).

In Belgium, data on a national level are available through a large scale mapping scheme. These data show that HA has become as common as the generalist species *Coccinella septempunctata* L., occurring in over 83% of the sampled area. Niche overlap analysis based on spatio-temporal overlap as well as plant use similarity shows that HA has a high degree of niche overlap with native species, especially with generalist and tree dwelling coccinellids in Belgium, which constitute about 50% of the native macroladybird (Coccinellinae, Epilachninae, Chilocorinae) fauna (Adriaens *et al.*, 2008). Preliminary analysis for only a few ladybird species has been performed based on 2000-2008 data of proportional occurrence (defined as the number of grid cells with species x in year y, divided by the number of sampled squares in year y). The results show that the expansion in range of HA coincides with a range decline in some native species, notably the generalist *A. bipunctata* and the deciduous tree species *A. decempuncata*. We detected a decrease of 40% in collection events (defined as the unique combination of date, observer and grid cell) with *A. bipunctata* and a decrease of 80% in the percentage of *A. bipunctata* observations in less than 10 years (2001-2008) on a national scale.

We hypothesize that intraguild predation may be one important mechanism for species displacement in the native ladybird guild based on the high degree of niche overlap with HA in species that are showing a range decline. Moreover, we hypothesize that *H. axyridis* may further expand its range in Europe and that an ongoing increase in HA abundance might eventually lead to local species extinction.

Future work will concentrate on analysing trends in native ladybird species before and after establishment of *H. axyridis* and verifying whether the reported changes in native ladybird community structure in Belgium are consistent throughout Europe. We aim at relating trends in native ladybirds to their ecology, in order to set up the profile of a native species sensitive to invasion by a potential intraguild non-native predator. This should be possible through enhanced international cooperation and joint analysis of available field data of well-known ladybird faunas (e.g. UK, Switzerland).

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What can endosymbionts tell about the *Harmonia axyridis* invasion?

Alexandre Aebi & Renate Zindel
Agroscope Reckenholz-Tänikon ART Research Station, Reckenholzstr. 191, 8046 Zürich, Switzerland.
Corresponding author: alexandre.aebi@art.admin.ch

Maternally-inherited endosymbiont bacteria are widespread among arthropod species (Hurst and Jiggins, 2000; Zchori-Fein and Perlman, 2004; Hilgenboeker *et al*., 2008). Recent studies on large samples of ladybirds showed that 52% of the species were infected with *Wolbachia, Rickettsia* or *Spiroplasma* (Weinert *et al*., 2007). To maintain themselves in their host population, these bacteria can dramatically alter their host's reproduction strategy by inducing parthenogenesis or cytoplasmic incompatibility, by feminization or by selectively killing infected males (O'Neill *et al*., 1997). Alternatively, an increasing number of studies show that endosymbionts can mediate pathogen resistance in their hosts (Brownlie and Johnson, 2009).

Understanding the factors determining the invasion success of alien species is a major challenge of invasion biology. *Harmonia axyridis*, with its widespread distribution and the rapidity, at which it expanded its geographic range, is the ideal system to study the role of intrinsic and extrinsic traits on invasive potential. Extensive human-mediated movement of *H. axyridis* poses limitations to population genetics methods (microsatellites) used to characterize its invasion routes (Lombaert *et al*., 2010). Sequence information of *Helicobacter pylori*, a chronic gastric pathogen of human beings was successfully used to answer unsolved questions about human migrations in the past (Falush *et al*., 2003). The great genetic diversity among bacterial populations and the limited linkage disequilibrium between polymorphic nucleotides within genes because of frequent recombination between bacterial strains (Baldo *et al*., 2006) suggest that sequence information of bacteria associated with insect species may provide valuable information on the host's population genetics.

In a collaborative project we are currently combining endosymbiont incidence and prevalence data with endosymbiont phylogenies to i) describe the endosymbiont community in the native and invaded range of *H. axyridis*, ii) assess the direction of spread of *H. axyridis*, iii) estimate the number of introduction events and iv) evaluate the role of endosymbionts on the invasive success of the Asian ladybird.

We believe that this study will shed light on the invasion success of *H. axyridis* and provide valuable information on the potential of endosymbionts as additional molecular tools.

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Position of *Harmonia axyridis* in aphidophagous guilds in the Czech Republic

Olga M. C. C. Ameixa[1,*], Alois Honěk[2], Zdenka Martinkova[2] & Pavel Kindlmann[1,3]
[1]Department of Theoretical Ecology, Institute of Systems Biology and Ecology AS CR and University of South Bohemia, České Budějovice, Czech Republic; [2]Research Institute of Crop Production, Drnovská 507, CZ 16106 Prague 6 – Ruzyně, Czech Republic; [3]Charles University, Benátská 2, Prague, Czech Republic.
**Corresponding author: olgameixa@portugalmail.pt*

Abstract: *Harmonia axyridis* was first recorded in the Czech Republic in 2006. Here we present an analysis of its degree of dominance within aphidophagous guilds and of its association with particular habitats in 2008. The dominance affinity of different ladybird species towards the habitats where they were found was studied using Canonical Correspondence Analysis (CCA). The biplot shows a clear invasive pattern in the year studied. In the present study, *H. axyridis* was one of the most abundant ladybirds, especially in trees and shrubs. Although it is too early to make firm conclusions about the role that this ladybird has on the native communities, it is worrying that it became one of the most abundant species only three years after its establishment in the Czech Republic.

Key words: *Harmonia axyridis*, diversity, abundance, habitat affinity, generation time

Introduction

The flightless strain of *H. axyridis* was introduced as a biological control agent into hop gardens in the northwest of the Czech Republic in 2003, but there have been no records of its establishment afterwards (Brown *et al.,* 2008). Therefore the main source of its individuals, which are increasingly reported from the Czech Republic since 2006 (first record exists from Prague O. Nedvěd and I. Kovář, unpublished data), is assumed to be its spread from Germany (Brown *et al.,* 2008). By 2008, *H. axyridis* became widespread everywhere throughout the Czech Republic.

Although *H. axyridis* is usually assumed to be semi-arboreal (e.g., Hodek, 1973), it occupies many habitats, and it has been recorded in meadows, heathlands and riparian zones (Adriaens *et al.,* 2008), reedbeds (Ware *et al.,* 2005; Brown *et al.,* 2008) and crop systems both in its native (Komai & Hosino, 1951) and recently colonized regions (Colunga-Garcia and Gage, 1998).

Harmonia axyridis is generally considered bivoltine in much of Asia (e.g., Osawa, 2000; Sakurai *et al.,* 1992), North America (Koch & Hutchison, 2003; LaMana & Miller, 1996), and Europe (Ongagna *et al.,* 1993). However, sometimes up to four to five generations per year have been observed (Wang, 1986, Katsoyannos *et al.,* 1997). The purpose of this work was to examine the relationship of *H. axyridis* with different types of habitats surveyed and weighing its presence regarding other native ladybirds.

Material and methods

In 2008 a survey was conducted in several types of habitats (wild herbs, trees and shrubs, and crops) in the South Bohemian region, Czech Republic. Adult coccinellids were collected using sweep-nets. The individuals were determined and released at the end of the collecting session in order not to be captured more than once. The duration of sweeping in each habitat was recorded, and since it differed due to differences in the sizes of the habitats, the number of individuals captured was recalculated to 30 minutes per each habitat and log transformed.

A total of 56 sites were visited 5 times along the season (from May to September), 40 of these sites were located in the Ceske Budejovice (48°58′ N, 14°28′ E) region and the other 16 in the surroundings of Cesky Krumlov (48°48′ N, 14°20′ E). Three of these habitats were excluded because there were no individuals collected there. Coccinellids were collected at 37 sites from trees and shrubs, at 10 sites from wild herbs and at 6 sites from crops. As wild herbs we understand herbaceous plants, without human intervention. In this category we swept *Urtica* sp., *Matricaria* sp., and ruderals with different plant composition. Among the trees and shrubs we included: *Malus* sp., *Salix* sp., *Acer* sp., *Rose* sp., *Betula* sp., *Prunus padus*, *Fagus* sp., *Quercus* sp., *Tilia* sp., *Salix* sp., *Populus* sp., *Sambuca nigra*, *Carpinus* sp. The survey on crops was made on: *Triticum* spp., *Medicago sativa*, and *Brassica napus*. The diversity and relative abundance of coccinellids within the habitats was compared. The differences in the diversity were obtained by the exponents of the regression curve, since they are positively correlated with diversity. Canonical correspondence analysis (CCA) was used, with habitat and year as response variables. Percent variance explained by the model was calculated by dividing the sum of canonical eigenvalues by the total inertia.

Results and discussion

Species diversity in the pooled sample

The native species *Coccinella septempunctata* (38%), *Propylea quatuordecimpunctata* (15%), *Adalia bipunctata* (14%) and *decempunctata* (11%), *Calvia quatuordecimguttata* (6%) and *Oenopia conglobata* (4%) were still more abundant than *H. axyridis*, whose relative abundance was only 4% (Figure 1).

Analysis of the species captured in each of the habitats separately revealed differences compared to the pooled sample of ladybird species.

Species diversity in crops

The communities of crops were dominated by *C. septempunctata* (82%), *P. quatuordecimpunctata* (13%), *A. decempunctata*. (2%). *Harmonia axyridis* (2%) was the third to fourth species in the rank of abundance (Figure 2).

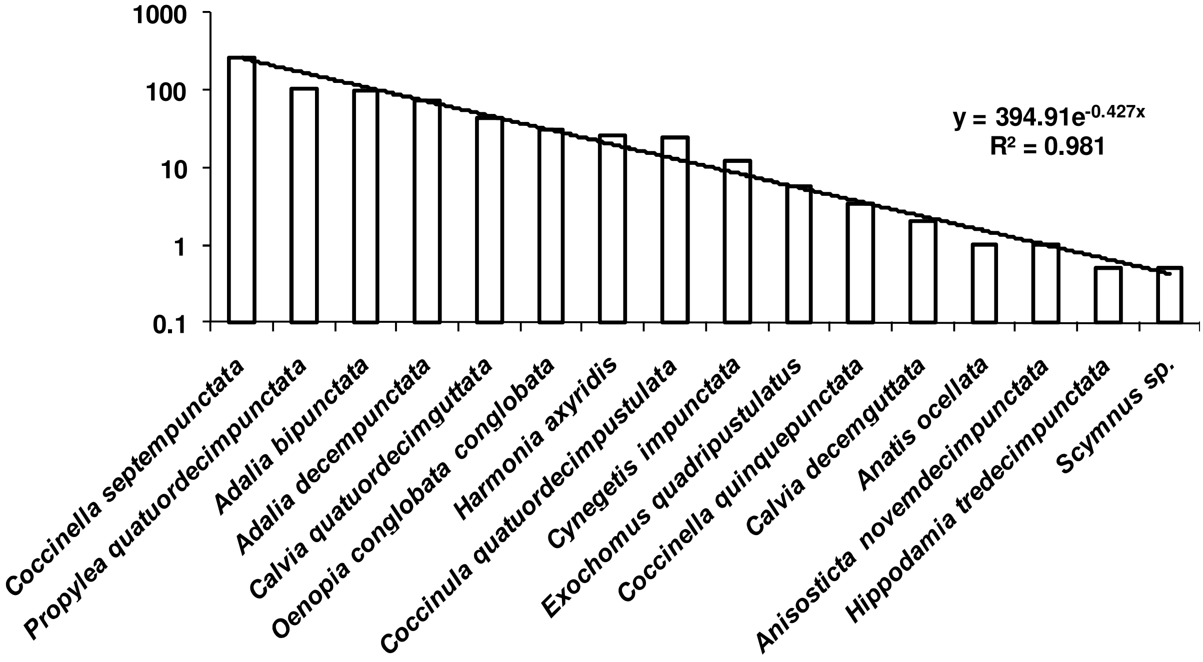

Figure 1. Coccinellid species diversity in all studied habitats in Czech Republic.

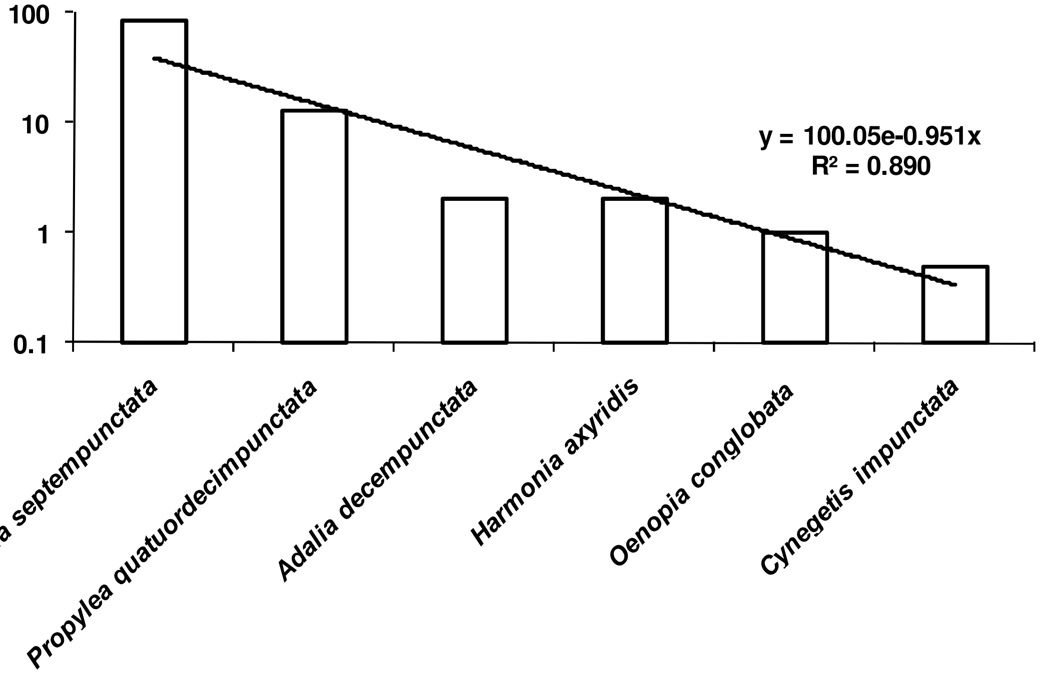

Figure 2. Coccinellid species diversity in crop habitats in Czech Republic.

Species diversity in wild herbs

The wild herb communities were dominated again by *C. septempunctata* (63%), *P. quatuordecimpunctata* (19%), *Coccinula quatuordecimpustulata* (8%), *Cynegetis impunctata* (5%). *Oenopia conglobata*, *A. bipunctata* and *A. decempunctata*, *Exochomus quadripustulatus* and *H. axyridis* were in the same order of abundance (around 1%) (Figure 3).

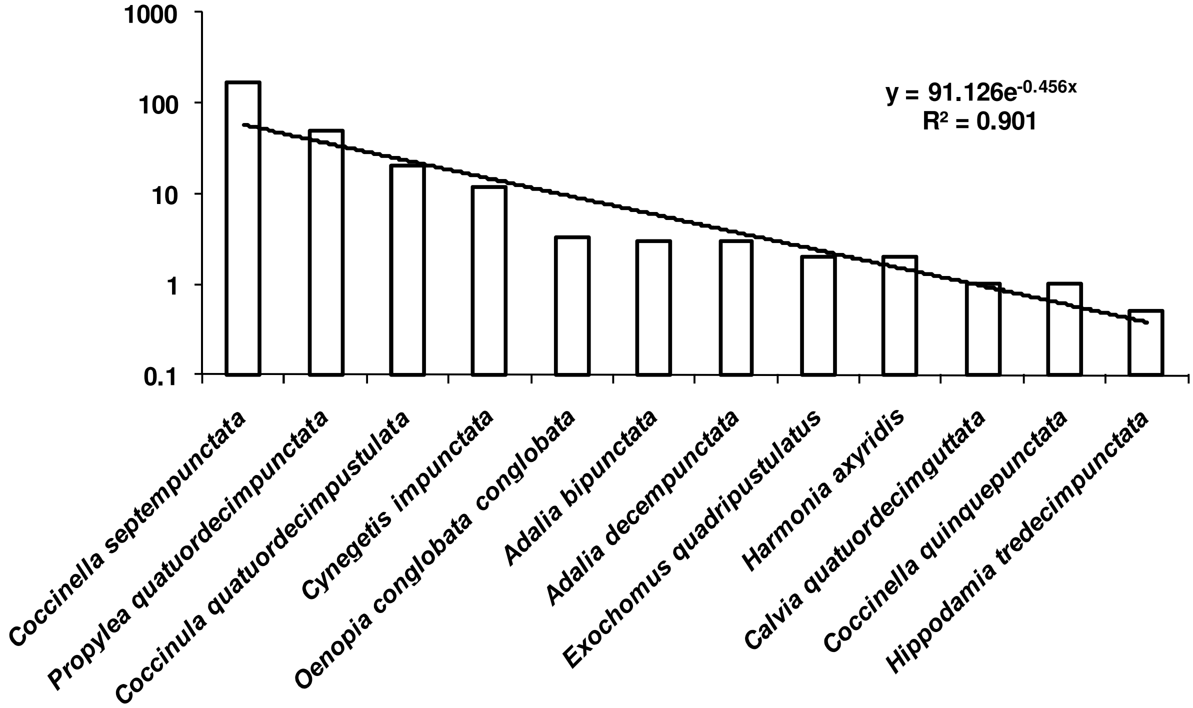

Figure 3. Coccinellid species diversity in wild herb habitats in Czech Republic.

Species diversity in trees and shrubs

Tree and shrub communities were dominated by *A. bipunctata* (30%), *A. decempunctata* (21%), *C. quatuordecimguttata* (13%), *P. quatuordecimpunctata* (13%), and *O. conglobata* (8%). *Harmonia axyridis* was the sixth in the rank of species abundance (7%) (Figure 4).

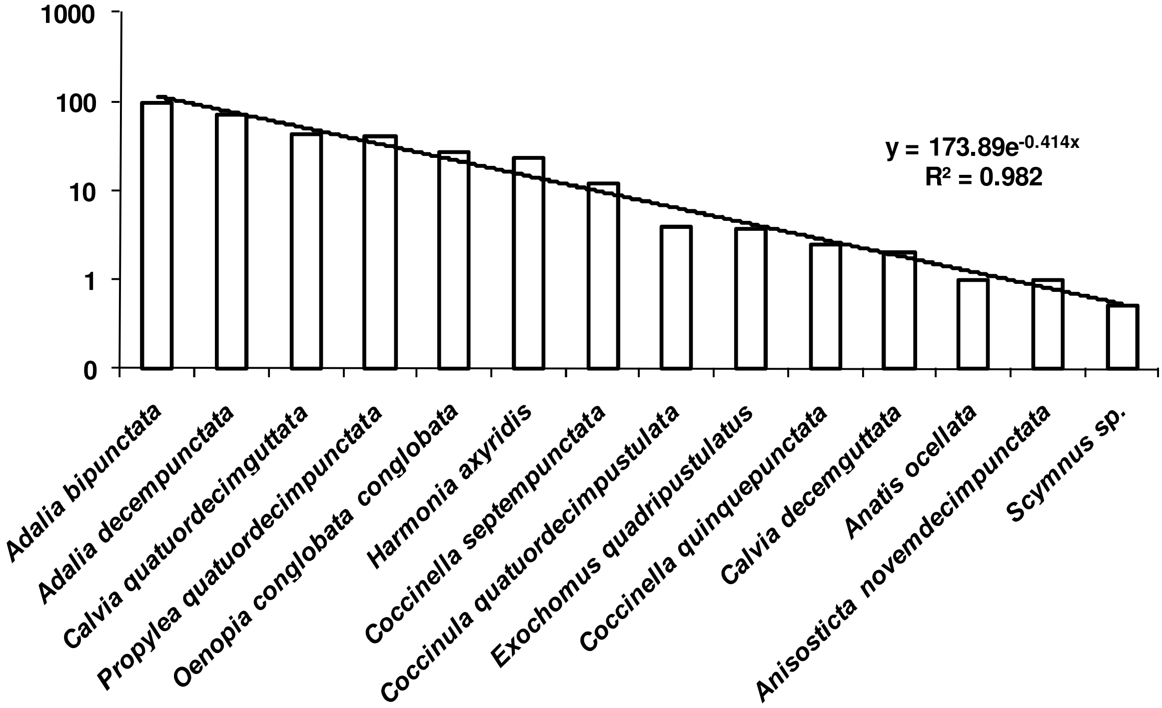

Figure 4. Coccinellid species diversity in tree and shrub habitats in Czech Republic.

Habitat association of the different species in South Bohemia

The biplot of the CCA is shown in Figure 5. Species are represented by grey dots (see caption for species names), the environmental variables, different habitats (crops, trees, wild herbs), are represented by grey arrows and labels. Although the model explains only 15.3% of variation (the sum of all canonical eigenvalues was 0.485, with the largest two being 0.444 and 0.041, respectively for the first and second axis, and the total inertia was 3.165), both axes are significant according to the Monte-Carlo test ($P = 0.002$).

According to the biplot, some species seem to be quite strongly associated with a particular habitat, especially in herbaceous and arboreal habitats. Thus species like *A. bipunctata* and *A. decempunctata* or *Calvia decemguttata* and *C. quatuordecimguttata* were associated with trees and shrubs and *C. septempunctata* and *P. quatuordecimpunctata* were more associated with crops and herbs.

Comparison of the three types of habitats shows differences between herbaceous and arboreal strata. *C. septempunctata* and *P. quatuordecimpunctata* are the two dominant species in the former, while various species of *Adalia* dominate the latter. The CCA analysis also indicates that some species are associated with a particular habitat.

The differences in the diversity can be conveniently expressed by the exponent of the regression curve, which is positively correlated with diversity: tree communities are most diverse (-0.414), followed by wild herb communities (-0.456), and crop communities (-0.951).

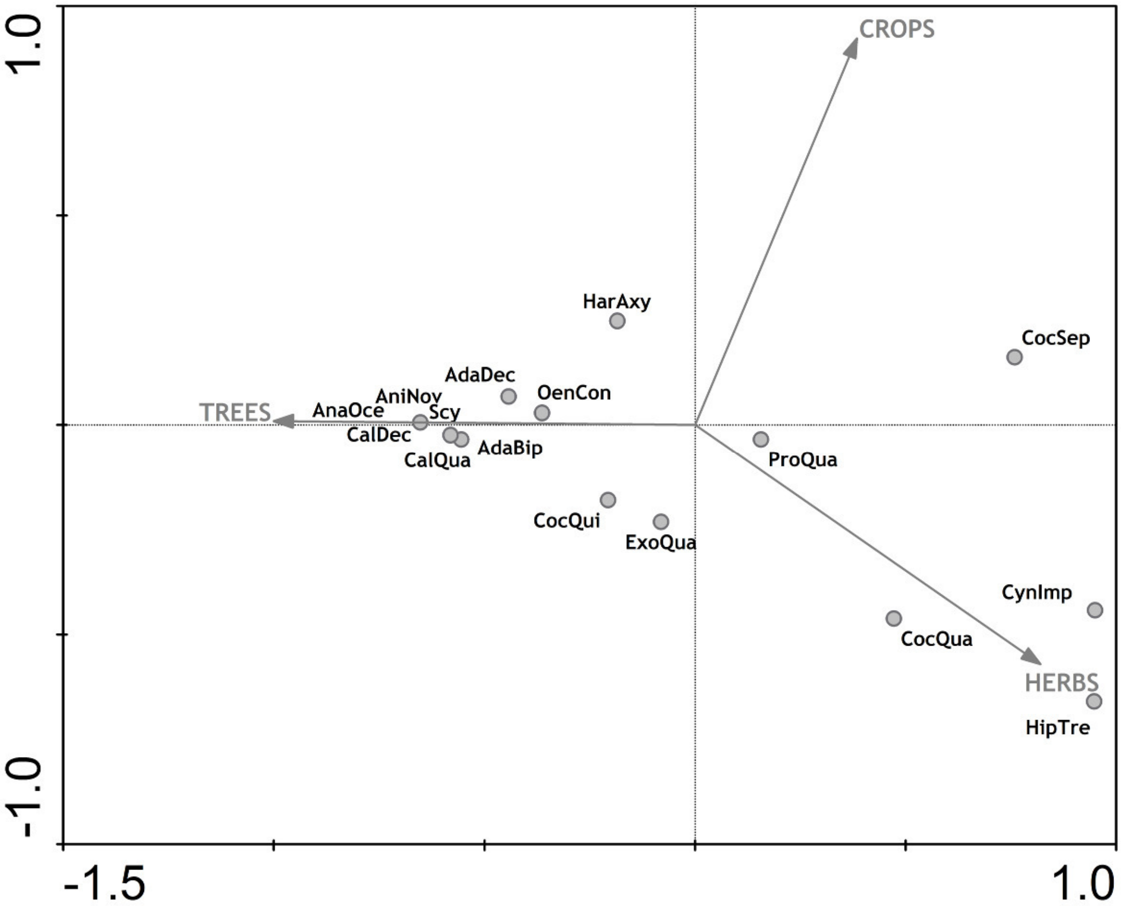

Figure 5. Canonical correspondence analysis of the cocinellid species community in Czech Republic, using habitats as a response variable.
Adalia bipunctata (AdaBip), *Adalia decempunctata* (AdaDec), *Anatis ocellata* (AnaOce), *Anisosticta novemdecimpunctata* (AniNov), *Calvia decemguttata* (CalDec), *Calvia quatuordecimguttata* (CalQua), *Coccinella quinquepunctata* (CocQui), *Coccinella septempunctata* (CocSep), *Coccinula quatuordecimpustulata* (CocQua), *Cynegetis impunctata* (CynImp), *Exochomus quadripustulatus* (ExoQua), *Harmonia axyridis* (HarAxy), *Hippodamia tredecimpunctata* (HipTre), *Oenopia conglobata* (OenCon), *Propylea quatuordecimpunctata* (ProQua), *Scymnus* (Scy).

Although *H. axyridis* is present in all types of habitats, it is relatively more abundant on trees, which suggests that they may be more suitable for this species. It is possible that this can be related with the fact that aphids are present for longer period on trees and shrubs than on wild herbs and crops. From the collection of larvae during 2009 (Ameixa & Kindlmann, unpublished data) we have some evidence that *H. axyridis* has more than 1 generation per year.

Other species that mainly live on trees, like *A. bipunctata*, can sometimes have overlapping generations (Hemptinne, 1989) and in warmer climates two or even three generations a year were observed (Lus, 1947). Apparently, the number of generations *per se* is not the only factor determining *H. axyridis* success, but probably a combination of this with intraguild predation, higher survival rate or stronger competitive skills can make *H. axyridis* so successful in invading new habitats.

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Overwintering ability of European populations of *Harmonia axyridis*

Nick Berkvens[1], Jeff S. Bale[2], Dirk Berkvens[3], Luc Tirry[1] & Patrick De Clercq[1]
[1]Ghent University, Department of Crop Protection, Laboratory of Agrozoology, Coupure Links 653, B-9000 Gent, Belgium; [2]University of Birmingham, School of Biosciences, Edgebaston, Birmingham, B15 2TT, UK; [3]Department of Animal Health, Prince Leopold Institute of Tropical Medicine, Nationalestraat 155 - B-2000 Antwerpen, Belgium

If an invasive species is to establish in a temperate or cold area it is imperative that it can endure the cold temperatures occurring during winter in that area (Bale, 1987, 1993, 1995). This is accomplished by means of overwintering strategies and cold survival (Labrie *et al.*, 2008). In the case of *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae), adults move to concealed and sheltered locations during winters, where they aggregate, hence, creating a protective microclimate with less extreme temperatures than in the surrounding area. While adults of *H. axyridis* overwinter in cracks and crevices of rocks, concrete buildings, caves or sometimes in leaf litter in the insect's natural range (Obata, 1986; Sakurai *et al.*, 1993), the species frequently overwinters in houses or other artificial structures in invaded areas. If aggregations in houses are large, the species can become a nuisance for their inhabitants (Kidd *et al.*, 1995; LaMana and Miller, 1996; Nalepa *et al.*, 1996; Yarbourgh et al., 1999; Huelsman *et al.*, 2001). Cold tolerance studies are considered a crucial step when assessing the establishment potential of biological control agents in the framework of a risk analysis (van Lenteren et al., 2003; Bazzocchi *et al.*, 2004; Tullett e*t al.*, 2004; Hatherly *et al.*, 2005a,b, 2008; van Lenteren *et al.*, 2008). Watanabe (2002) and Koch *et al.* (2004) have studied the cold tolerance of overwintering populations of *H. axyridis* in Japan and the USA, respectively. The present study investigated the cold tolerance of the invading populations in Europe and assessed the overwintering ability of *H. axyridis* as a function of hibernaculum type.

From November 2007 to February 2008, individuals were monthly collected in an outdoor and indoor hibernaculum. The outdoor hibernaculum consisted of spaces along the doorframes of an old deserted bunker located in the middle of the nature reserve in Wilrijk, Belgium (referred to as Population A). The indoor hibernaculum consisted of spaces along window frames of an unheated stairwell at the southwest wing of a mansion, situated in the middle of a large park in Gaasbeek, Belgium (referred to as Population B), respectively. The cold hardiness of the insects was evaluated by determining the supercooling point (SCP, i.e. the temperature at which the body fluids freeze), lower lethal temperature (LTemp50, i.e. the temperature at which 50% of the test individuals die) and lower lethal time at -5 and 0°C (LTime50, i.e. the time required to kill 50% of the population at a certain temperature), using methods described in Watanabe (2002), Hatherly *et al.* (2004, 2005a,b, 2008), Koch *et al.* (2004) and Tullett *et al.*(2004).

Throughout winter, SCP and LTemp50 varied around -16.5°C for adults in the outdoor hibernaculum and were always lower than -14°C for those in the indoor hibernaculum (Berkvens *et al.*, 2009). Furthermore, since the beetles usually aggregate as to create a protective microclimate when overwintering (Koch *et al.*, 2004), they can possibly survive in environments with even lower subzero temperatures. The LTime50 was always higher than 4 and 4.5 months at -5 and 0°C, respectively (Berkvens *et al.*, 2009). These results indicate that the European populations of *H. axyridis* are sufficiently cold hardy to enable establishment in large parts of the continent. In addition, this study suggests that the lethal time is a more reliable cold tolerance parameter since the experimental procedure resembles the conditions experienced by insects during temperate winters more closely.

In contrast to outdoor overwintering individuals maintaining a consistent cold tolerance during winter, that of individuals overwintering in more sheltered hibernacula decreased as winter progressed (Berkvens *et al.,* 2009). Further research is needed to investigate if this decrease in cold tolerance would trigger an early activation of these individuals. If this is the case, a premature activation at times when food is absent could lead to a higher mortality for individuals overwintering indoors. The fact that adults cause a nuisance during their overwintering in houses (Yarbourgh *et al*., 1999; Huelsman *et al*., 2001), could partially be a consequence of their search for food indoors due to early activation. Morph type and sex had no influence on the cold hardiness of *H. axyridis*.

In addition, the cold tolerance of field collected adults was compared to that of adults taken from laboratory populations. A non-melanic and melanic population were established in the laboratory using insects collected in a park in Ghent, Belgium, which were then further reared according to methods described in Berkvens *et al*. (2008 a,b). The cold tolerance of newly emerged adults of the 3rd laboratory generation of both populations was measured. All cold tolerance parameters differed greatly between adults of these laboratory populations and the field collected adults of populations A and B (Berkvens *et al*., 2009). This cautions for a hasty extrapolation of conclusions based on cold tolerance experiments with laboratory-reared individuals, given that these may not be representative for field populations.

In conclusion, this study indicates that the strong cold hardiness of *H. axyridis* may aid in the establishment of the coccinellid in large parts of Europe.

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Looking beyond the spots: inspiring the public to record ladybirds

Peter M. J. Brown, Remy L. Ware & Helen E. Roy
Department of Life Sciences, Anglia Ruskin University, East Road, Cambridge, CB1 1PT, UK; Department of Genetics, University of Cambridge, Downing Street, Cambridge, CB2 3EH, UK; NERC Centre for Ecology & Hydrology, Benson Lane, Crowmarsh Gifford, Oxfordshire, OX10 8BB, UK
Corresponding author: peter.brown@anglia.ac.uk

The UK has a long tradition of biological recording and a highly developed infrastructure for processing species data. Established in 1964, the Biological Records Centre (www.brc.ac.uk) is the hub of this infrastructure, listing 85 national recording schemes for lower and higher plants, invertebrates and vertebrates. The Coccinellidae Recording Scheme is one such scheme. Launched in 1968, it collates and organises records of the UK's 46 coccinellid species, and maps their distributions.

The Coccinellidae Recording Scheme was led by Professor Michael Majerus (hereon referred to as Mike) from the 1980s. In the early years, participants tended to be experienced naturalists. To draw others in, Mike set up the very successful, public participatory, Cambridge Ladybird Survey (CLS), that ran from 1984 to 1994. As a way of making the coccinellid species more accessible to non-entomologists, Mike allocated common names to the conspicuous species. This was a small but important step; members of the UK public may not have been able to relate to *Exochomus quadripustulatus* (L.) (Coleoptera: Coccinellidae), but were much more likely to relate to the pine ladybird, the common name for the same species.

The CLS generated a huge amount of data and made a significant contribution to Mike's New Naturalist 'Ladybirds' book (Majerus, 1994). For example, new information on overwintering habitats, phenology and inter-generational matings of coccinellids were contributed. Detailed and informative newsletters were produced, usually twice a year, to share interesting findings received by the survey, along with Mike's insights and summaries of some of his research. Ten years before *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) arrived in Britain, Mike included drawings of some of the many colour forms of this highly polymorphic species in one of the newsletters. At the end of the CLS, a provisional distribution atlas for the conspicuous coccinellid species was produced (Majerus, 1995). This is currently the only atlas of UK coccinellid species (although the UK Ladybird Survey team are in the process of writing a new atlas).

When *H. axyridis* arrived in the UK in late 2004, Mike realised the potential for involving the public in a unique opportunity to study the spread of an invasive animal from the start of the invasion process. Funding was applied for and quickly granted by the UK Department for Environment, Food and Rural Affairs (Defra – www.defra.gov.uk) via the National Biodiversity Network Trust (www.nbn.org.uk). Collaborative work between Mike, Helen Roy and staff at the Biological Records Centre enabled the Harlequin Ladybird Survey (HLS – www.harlequin-survey.org) to be launched in early 2005 at the Natural History Museum, London. It was one of the first online wildlife surveys in the UK. Soon afterwards, a re-branded Coccinellidae Recording Scheme, the UK Ladybird Survey (UKLS – www.ladybird-survey.org), was launched.

The HLS/UKLS team were very active in terms of public outreach and over 100 talks and events were delivered from 2005 to date. More than 26,000 online records of *H. axyridis* have been received, enabling a study of unprecedented detail. The spread of the species was recorded at high resolution, and calculations on the rate of spread (approximately 100km per year) (Brown et al., 2008) made. Ten km square records on a UK map were the broad-scale results of the HLS, but far more detailed insights into the ecology of *H. axyridis* were possible. For example, photographs of *H. axyridis* consuming the eggs and larvae of Lepidoptera were received, and further work in this area is underway (Wells et al., 2009). Many other studies have arisen as a direct result of the HLS. These include a study on the impact of *H. axyridis* on native coccinellids (Brown et al., in prep.), genetic work to discover if *H. axyridis* is feeding on *Adalia* species in the wild (Thomas et al., 2009a), genetic work on the origins of *H. axyridis* in its introduced range (Thomas et al., 2009b), laboratory studies on intraguild predation (Ware & Majerus, 2008), ecological modelling of survey data (Comont et al., 2009) and work on natural enemies (Koyama & Majerus, 2008; Rhule et al., 2009; Roy et al., 2008). A UK project to involve the public in recording a suite of non-native animals and plants is launching in 2010, and is modelled on the HLS. This will be available via the GB Non-Native Species Secretariat (www.nonnativespecies.org).

Effective use of webpages and online recording enables the HLS to deal efficiently with large volumes of data, and to verify submitted records quickly. Recorders are asked to include a photograph of the coccinellid recorded, if possible, and to upload this to the HLS online recording form. This is enabled by high levels of access to the internet and digital photography, and advances in technology (making good digital cameras increasingly affordable to the public). Providing feedback to recorders is seen as important, so an automated email response system is in place. Thus when a coccinellid record is verified, an email is sent to thank the recorder for participating in the survey, inform them of the identification of their coccinellid and give further information. This is an effective way of encouraging further involvement. The HLS and UKLS continue to be extremely active. A special exhibit initiated by Mike at the prestigious Royal Society Summer Science exhibition in 2009 (www.summerscience.org.uk/09) was a fitting tribute to his coccinellid career.

Mike was very adept at using the media to convey his message. His charisma and eye for a story led to many television and radio appearances. When the CLS was running, Mike appeared on BBC TV's Blue Peter, a very popular children's programme, leading to many extra contributions of coccinellid data. Mike worked and filmed with many of the UK's most famous naturalists, including David Attenborough and Bill Oddie. Recently the HLS was featured on BBC TV's SpringWatch and AutumnWatch. All of the UK's national newspapers have featured articles on *H. axyridis* (repeatedly in some newspapers). Articles written for popular science journals such as British Wildlife (Roy et al., 2005) and BBC Wildlife (Brown, 2007) further helped to raise the profile of the surveys. Publication of an inexpensive and widely available laminated field chart (Majerus et al., 2006) showing all of the conspicuous UK coccinellids, helped recorders with their identifications.

The clear advantage of involving the public in the coccinellid surveys is the potential for generating many times the volume of data that a small research group could collect. Informing the public of important environmental issues, using ladybirds as a model, is a further benefit. For example, questions such as 'Why do invasive species matter?' can be addressed. Coccinellids, partly because of the relatively small number of UK species, are seen as more straightforward to identify than many other families in the Coleoptera, and thus may be regarded as a good introduction to the study of beetles. It is hoped that enthusiasm for other taxa may then be stimulated in some recorders. Data quality issues are overcome by the request for photographs or specimens to back up records from non-experts. In this way approximately 40% of the records received for *H. axyridis* are able to be verified.

Mike's expertise extended far beyond the Coleoptera, and he had a particular passion for the Lepidoptera. He was well known for his evolutionary studies of the peppered moth *Biston betularia* (L.) (Lepidoptera: Geometridae). He authored a further volume of the highly respected New Naturalist series, 'Moths' (Majerus, 2002). Mike joined the Amateur Entomologists' Society as a teenager and was president from 2005 until his untimely death in 2009. His presidency enabled further outreach opportunities and he was an inspiration to many, perhaps especially the younger members of the society.

Key words: Biological recording, Coccinellidae, outreach, public surveys, science & society

Acknowledgements

This paper is dedicated to Professor Michael Majerus, our friend and colleague. Much of what we know about coccinellids, we learnt from Mike. We hope that we can take some of his knowledge and enthusiasm into our future work. We thank everybody who has contributed records to the various ladybird recording schemes.

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Is *Harmonia axyridis* a marginal host for the parasitoid wasp *Dinocampus coccinellae*? Morphological and molecular genetic analyses

Joseph M. Burling[1], Remy L. Ware[2] & Lori Lawson Handley[1*]
[1]Evolutionary Biology Group, Department of Biological Sciences, University of Hull, HU6 7RX, UK; [2]Department of Genetics, University of Cambridge, Downing Street, Cambridge, CB2 3EH, UK
Corresponding author: l.lawson-handley@hull.ac.uk

Abstract: It has previously been suggested that *Harmonia axyridis* is a marginal host for the parasitoid wasp, *Dinocampus coccinellae*. To investigate this further, we performed morphological and molecular genetic analyses of wasps from Japanese and UK populations of *Coccinella septempunctata* and *H. axyridis*. Wasps that emerged from *H. axyridis* were smaller than those from *C. septempunctata*, and there was no genetic evidence of host specialisation. Our data therefore supports the hypothesis that *H. axyridis* is a marginal host, which has implications for natural and biological control of this invasive species.

Key words: Coccinellidae, *Dinocampus coccinellae*, *Harmonia axyridis*, natural enemies, parasitoid wasp

Introduction

The parasitic mode of life is very common among insects, and has evolved in many arthropods. There are estimated to be around 225,000 species of parasitoid wasps, many of which infect only one or a few suitable hosts (Schmidt *et al.,* 2001). A host is defined as 'suitable' if it allows all or most individuals to complete development, whereas an unsuitable host does not allow any parasitoids to complete development (Heimpel *et al.,* 2003). Meanwhile, a 'marginal' host allows only a small proportion of parasitoids to develop. However, some species of parasitoid are known to readily oviposit within a marginal host, both in the laboratory and field, even in the presence of a suitable host (Heimpel *et al.,* 2003).

Dinocampus coccinellae (Hymenoptera: Braconidae), a solitary endoparasitoid wasp, infects coccinellids of the sub-family Coccinellinae, and has been reported in over 50 species throughout the Holarctic region (Koyama & Majerus, 2008). In Japan, *D. coccinellae* has been reported attacking several species, including *Coccinella septempunctata brucki* and *Harmonia axyridis* (Koyama & Majerus, 2008). In the UK, *C. septempunctata* is recognised as the most common host, with particularly high rates of infection reported from overwintering samples (Majerus, 1994). Recent evidence suggests that *D. coccinellae* is starting to attack *H. axyridis* as a novel host in the UK (Ware *et al.,* 2010). However, it is thought that *H. axyridis* is a marginal host for *D. coccinellae* in both the native and introduced ranges (Firlej *et al.,* 2005; Hoogendoorn & Heimpel, 2003). In laboratory experiments, *D. coccinellae* showed a higher rate of emergence relative to ovipositional attack from *C. septempunctata* then from *H. axyridis* for both Japanese and UK wasps (Koyama & Majerus, 2008).

In this study, we aimed to further investigate the hypothesis that *H. axyridis* is a marginal host for *D. coccinellae* using morphological and genetic analyses. Development is expected to be impaired in a marginal host and there is evidence that female reproductive output is positively correlated with body size in insects (Gotthard *et al.,* 2007; Bauerfeind & Fischer, 2008). Therefore, we predicted that, relative to host size, wasps emerging from *H. axyridis* will be smaller than those emerging from *C. septempunctata*. If sub populations of a parasite specialize on different hosts, this can lead to genetic differentiation and eventual speciation. We therefore predicted that there should be no genetic evidence of host specialization by *D. coccinellae* in the host's native range.

Material and methods

D. coccinellae from four different host types were analyzed: UK *C. septempunctata* (n=56), Japanese *C. septempunctata brucki* (n=16), UK *H. axyridis* (n=8) and Japanese *H. axyridis* (n=9).

Morphological measurements

Segments of the wasps' left hind leg (femur, tibia, first tarsal and second tarsal), and the ovipositor were accurately measured using a stereo microscope. The maximum length of the host elytra was also determined, and was treated as a covariable during analysis (Analysis of Covariance, ANCOVA).

Genetic analysis

DNA from each wasp was amplified via PCR and sequenced at mtDNA *COI*. DNA sequences were aligned and a Neighbour Joining tree constructed using MEGA 4.0.1 (Tamura *et al*., 2007).

Results and discussion

D. coccinellae emerging from *H. axyridis* had significantly shorter femurs (ANCOVA F=16.5, $p<0.001$) and tibias (ANCOVA F=11.7, $p<0.01$) than those emerging from *C. septempunctata* or *C. septempunctata brucki*. This was seen even though there was no significant difference observed between the elytra length of different hosts (ANOVA F=0.396, $p>0.05$). In addition, there was no evidence for host specialisation, as wasp mtDNA haplotypes were shared between different host types. These results are consistent with the hypothesis that *H. axyridis* is a marginal host for *D. coccinellae*. Further work is needed to investigate adaptation of *D. coccinellae* to *H. axyridis* in the host's invasive range.

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Modeling *Harmonia axyridis* (Coleoptera, Coccinellidae) interactions within the aphidophagous guild

Richard F. Comont[1, 3]*, Richard Harrington[2], Owen T. Lewis[3], Beth V. Purse[4] & Helen E. Roy[1]

[1]NERC Centre for Ecology & Hydrology, Wallingford, UK; [2]Rothamsted Research, Harpenden, UK; [3]University of Oxford, Oxford, UK; [4]NERC Centre for Ecology & Hydrology, Edinburgh, UK.

** Corresponding author: rimo@ceh.ac.uk*

The rapid spread of the invasive alien coccinellid, *Harmonia axyridis* (Pallas), across the British Isles is predicted to impact native coccinellids through direct competition and intra-guild predation (Majerus *et al*., 2006; Pell *et al*., 2008). Additionally, the population dynamics and seasonal overlap of coccinellids with their aphid prey is likely to be affected by climate change (Skirvin *et al*., 1997). The combined effects of these two factors could alter relationships within the aphidophagous guild.

A wide range of habitat-, prey-, and climate-related traits, along with intrinsic attributes, have been collated from the literature for the 26 larger ladybird species resident in the British Isles. This subset of the UK coccinellid fauna (total 46 species) was chosen as they are the targets of a very active recording scheme (Brown *et al.,* 2010) and are relatively easily identified in the field, resulting in large volumes of high-quality occurrence data. This dataset of traits from the literature will be used to quantify the niche overlap of each of the 25 native species with *Harmonia axyridis*, particularly in terms of prey, habitat, and phenology.

These niche characteristics will be analysed with a range of datasets (including land use and climate data, aphid abundance data from the Rothamsted Insect Survey (RIS) suction trap network, and ladybird occurrence records from the RIS and from the Biological Records Centre (BRC) databases, comprising the UK and Cambridge ladybird surveys) to quantify the relative roles of top-down, bottom-up and abiotic factors in driving native coccinellid dynamics.

Time-series analysis will be applied to long-term RIS and BRC datasets, and, in the light of the previous analysis, the relative roles of changes in climate, aphid prey abundance, and *H. axyridis* occurrence in driving annual changes and longer-term trends in the abundance of native coccinellids will be examined.

These modelling initiatives will be combined with, and tested by, mesocosm experiments investigating coccinellid/coccinellid and coccinellid/prey interactions in the presence of different aphid densities and aphid species of varying palatabilities. The ultimate aim is to quantify responses of native coccinellids to *H. axyridis*, accounting for the differing specialisations of the species. In particular, the functional response of *H. axyridis* to the abundance of aphids and potential prey species of coccinellids will be investigated, guided particularly by any changes in coccinellid community composition with differing levels of *H. axyridis* invasion suggested by the niche overlap and time-series analyses.

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Ladybird beetle invasions: traits of invasive species

A. F. G. Dixon[1] & J.-L. Hemptinne[2]
[1]Institute of Systems Biology, Academy of Sciences, Ceske Budejovice, Czech Republic and School of Biological Sciences, University of East Anglia, Norwich, UK; [2]Université de Toulouse, Castanet-Tolosan, France
Corresponding author: a.f.dixon@uea.ac.uk

The terminology used in the burgeoning literature on invasive species does not consist of acronyms of the names of the various processes or structures as in other aspects of biology but in emotive terms, which we found more confusing than helpful. In Table 1 is a list of the most frequently used terms and their ecological equivalents. Why it is necessary to create a separate jargon when well tried and understood terms exist in the ecological literature is puzzling but will not be discussed further here.

Table 1. Terminology

Emotive	**Ecological equivalent**
Alien	Non-native
Impact	Effect
Invade	Disperse
Invaders	Dispersing organisms
Invasion	Spread
Invasiveness	Rate of spread
Propagule pressure	Reproductive rate
Retreat	Decline in abundance

The aim of this paper is to analyze the information in the literature on the rate of spread of non-native ladybirds in order to determine whether any feature(s) of the life history traits of so called invasive species can account for their rate of spread and abundance. For this we used the extensive and detailed literature on the introduction and spread of non-native ladybirds in North America. These ladybirds were either accidentally or intentionally introduced, and because they feed on pest aphids and coccids were initially regarded as beneficial organisms. Of these species two, *Coccinella septempunctata* and *Harmonia axyridis*, were particularly successful in terms of their rate of spread and current level of abundance. An analysis of their life history traits and that of another three non-native species that also became established and spread revealed that if ladybirds can survive introduction into a novel environment and there is an extensive suitable habitat for them to colonize, then they will become abundant if they feed on aphids and spread particularly rapidly if they are large. That is, body size is a good predictor of the potential "invasiveness" in aphidophagous ladybirds.

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Dynamics and impact of *Coccinella septempunctata* as another invasive ladybird beetle in North America

Edward W. Evans
Department of Biology, Utah State University, Logan, Utah, 84322-5305 USA
E-mail: ted.evans@usu.edu

Abstract: *Harmonia axyridis* is the second species of Coccinellidae recently to cause much alarm in North America as an invasive non-native species. The first was *Coccinella septempunctata*, which spread (both naturally and with human assistance) from the early 1970s through the early 1990s to attain high densities throughout North America. This establishment and rapid range expansion is striking; numerous attempts previously to introduce *C. septempunctata* to North America as a generalist biocontrol agent (classical biological control) seemingly had failed. I present a brief account here of *C. septempunctata* in North America, with focus on possible non-target effects beyond pest (aphid) suppression. I consider in particular ideas and evidence associated with four major mechanisms hypothesized to result in adverse effects on native coccinellids: exploitative competition among larvae, intraguild predation, habitat compression/shift, and interspecific hybridization. These mechanisms need to be evaluated further. The challenge has intensified with the establishment now of *H. axyridis*, which may join with *C. septempunctata* in having far reaching effects in North America.

Key words: Coccinellidae, competition, habitat selection, intraguild predation, non-target effects, species displacement

Introduction

Ladybird beetles (Coccinellidae) as generalist biocontrol agents have been introduced repeatedly to North America for many decades (Gordon, 1985). But American public sentiment concerning such efforts has changed in recent years. In particular, the establishment and spread of *Harmonia axyridis* (Pallas) throughout the continent has alarmed much of the general public as well as many entomologists and ecologists, as this ladybird has acted as much or more as a harmful invasive species than it has as a beneficial biocontrol agent (Koch & Galvan, 2008). To make matters worse, this species is the second "high-profile" invasive ladybird in recent years to sweep in breathtaking fashion across North America. It was preceded a decade earlier by *Coccinella septempunctata* L. (e.g., Louda *et al*., 2003). Interestingly, nascent populations of both ladybirds were discovered at distant locations from areas to which these species had been introduced previously with presumed lack of success, and both species thereafter quickly spread from these nascent populations to occupy large new ranges in North America (Schaefer *et al*., 1987; Tedders & Schaefer, 1994). Non-target effects of *H. axyridis* on other insect species, and the ecological mechanisms underlying these effects, have been subject to much recent investigation in North America (e.g., Cottrell & Yeargen, 1998; Michaud, 2002; Koch *et al*., 2005; Cottrell, 2007). As reviewed below (with apologies to authors whose work is credited only indirectly [i.e., as cited in references presented in this brief summary]), non-target effects and mechanisms have similarly been the subject of intensive investigation for *C. septempunctata*.

Establishment, spread, and present status

Efforts, seemingly unsuccessful, to introduce *C. septempunctata* to various locations in both eastern and western North America began as early as 1957 and continued through the early 1970s (Gordon, 1985; Wheeler & Hoebeke, 1995). But then in 1973, populations of this ladybird, perhaps established accidentally from transoceanic shipping (Day *et al*., 1994), were discovered in New Jersey (Angalet *et al*., 1979) and Quebec (Larochelle & Larivière, 1975). Over the next five years, more than half a million individuals of this species were redistributed throughout the United States, as far south as Florida and as far west as New Mexico and Utah (Angalet *et al*., 1979). By summer 1986, *C. septempunctata* was well-established throughout northeastern and midwestern states (Schaefer *et al*., 1987). Within five to ten years thereafter, it had spread throughout essentially all of North America.

As in the Old World (Hodek & Honěk, 1996), *C. septempunctata* reaches greatest numbers in herbaceous habitats in North America, but nonetheless it occurs in a great variety of semi-natural and natural habitats (e.g., old fields undergoing succession, shelterbelts, pine stands) as well as in agricultural crops (e.g., alfalfa, soybean, wheat, apple) (Maredia *et al*., 1992; Obycki *et al*., 2000; Hesler & Kieckhefer, 2008). It shows no sign of low genetic diversity (genetic bottleneck) associated with its colonization of North America (Krafsur *et al*., 2005). Particularly in agricultural crops such as alfalfa and potato, numbers of *C. septempunctata* rose very rapidly such that within a few years of its arrival, this species greatly outnumbered native North American ladybirds also inhabiting these crops (e.g., Evans 2000; Turnock *et al*., 2003; Alyokhin & Sewell, 2004). Although *C. septempunctata* was subsequently replaced by the later invading *H. axyridis* in apple (arboreal) habitat (Brown and Miller, 1998; Brown 2003), it has remained very abundant even as *H. axyridis* has established in herbaceous crops (Alyokhin & Sewell, 2004; Evans, 2004; Gardiner *et al*., 2009). At such high numbers, *C. septempunctata* is thought by many to have significant impact on pest aphid populations in North America, but an open question is the degree to which such impact is merely substituting for that of displaced native ladybirds (e.g., Elliott *et al*., 1996; Obrycki *et al*., 2000).

Why so successful?

What accounts for the great success of *C. septempunctata* in overtaking native North American species in abundance in these habitats? Hodek & Michaud (2008) stress its generalist habits and its broadly flexible life history and reproductive habits. Interspecific comparison revealed individuals within populations of this species to be more variable as adults in their size than individuals of several native North American species, perhaps reflecting that individuals of the invasive species matured under a greater variety of ecological conditions (Evans 2000). This species may also be especially well adapted to thrive and multiply in European-derived crops such as alfalfa, and to achieve high rates of reproduction and dispersal by virtue of its large body size and its production of large numbers of relatively small eggs (Hemptinne *et al*., in prep; Kajita & Evans, 2009a,b).

Non-target effects

From the beginning, concerns were expressed that the great success of *C. septempunctata* in reaching high numbers in North America might have unintended, adverse effects on non-target species. Horn (1991) raised the possibility that this ladybird might further endanger rare butterflies by feeding on them as immatures, a possibility investigated and supported by

Schellhorn *et al.* (2005) for the Karner blue butterfly (*Lycaeides melissa samuelis* Nabokov) in particular. Others raised concerns that *C. septempunctata* ironically might sometimes actually undermine biological control of aphids, by consuming parasitized aphids (i.e., by acting as an intraguild predator) and/or by adversely influencing adult parasitoid foraging behavior (e.g., Ferguson & Stiling, 1996; Taylor *et al.* 1998; Nakashima & Senoo, 2003). Based on field experimental results, Evans & England (1996) suggested that *C. septempunctata* also could undermine parasitism of insect pests other than aphids (e.g., the alfalfa weevil) by consuming aphids. Such consumption could deny these pests' parasitoids a source of adult nutrition (honeydew) particularly important in large monocultural crops largely devoid of other food sources for the parasitoids (especially floral nectar).

Effects on native North American ladybirds

As with *H. axyridis* (e.g., Snyder *et al.*, 2004; Yasuda *et al.*, 2004; Harmon *et al.*, 2007), most attention concerning non-target effects of *C. septempunctata* has focused on potential adverse consequences for native North American ladybirds (Simberloff & Stiling, 1996; Evans, 2000; Obrycki *et al.*, 2000; Louda *et al.*, 2003). Concerns were raised early on (e.g., Schaefer *et al.*, 1987). Two early reports linking population declines of native ladybirds during the 1980s and early 1990s with the invasion of *C. septempunctata* were especially influential. Wheeler & Hoebeke (1995) documented the coincidence in the dramatic decline of *Coccinella novemnotata* Herbst and the rapid rise in numbers of *C. septempunctata* throughout northeastern North America. Elliott *et al.* (1996) analyzed long-term census data of crops in eastern South Dakota, and found similarly dramatic declines in numbers of *Coccinella transversoguttata richarsoni* Brown and *Adalia bipunctata* (L.) associated with the establishment of *C. septempunctata* in those crops (in further assessment of autecologies and habitat overlap, Obrycki *et al.* (2000) judged the potential for displacement by *C. septempunctata* as high and widespread for *C. transversoguttata*, but more localized for *A. bipunctata*). Anticipating and following these and similar reports, experimental studies were conducted to investigate the ecological mechanisms driving species displacements. As summarized below, attention focused on four potential mechanisms: competition among larvae, intraguild predation, habitat shift (compression), and interspecific hybridization.

Competition among larvae

A hypothesized basic asymmetry favoring *C. septempunctata* over native ladybirds in exploitative, interspecific competition among larvae was not supported by initial laboratory experiments in which prey (aphid) availability was moderately high (Evans, 1991; initial prey numbers were set at moderate levels in these experiments to explore the importance of predator diversity for prey population control). Such asymmetry did arise in subsequent laboratory experiments in which prey numbers were set very low (Obrycki *et al.*, 1998a), but not in companion field cage experiments (conducted in plots of potatoes; Obrycki *et al.*, 1998b). Evans (2000) tested for increasing larval competition in field populations by comparing adult body size of native ladybirds among years, but found no trends of decreasing body size over time that might reflect diminishing access to food as *C. septempunctata* rapidly increased in numbers.

Intraguild predation

Obrycki *et al.* (1998a) noted that their results (asymmetry favoring *C. septempunctata* when paired with *Coleomegilla maculata* (De Geer) under very limited food conditions) could have arisen in part or largely from larval intraguild predation. Subsequent laboratory studies revealed that *C. septempunctata* was a relatively weak intraguild predator, especially in comparison with *H. axyridis*, in larval interactions with native ladybirds (Snyder *et al.*, 2004; Yasuda *et al.*, 2004; Moser & Obrycki, 2009). Nonetheless, it remains possible that by sheer numerical dominance, *C. septempunctata* as an intraguild predator may be, in some natural settings, an important mortality agent for native ladybirds (including as eggs).

Habitat shift (compression)

Evans (2000, 2004) hypothesized that population declines of native species in agricultural crops might reflect indirect interaction with the invasive *C. septempunctata* that influences these species' habitat selection (the habitat compression or habitat shift hypothesis (MacArthur & Wilson, 1967). Dispersing adults of native ladybird species appear especially responsive to varying aphid densities in alfalfa fields (Evans, 2004; Evans & Toler, 2007). As suggested by the low aphid numbers in these fields that have followed the establishment of *C. septempuncatata*, predation by this very abundant invader now may often substantially dampen aphid population growth, thereby undermining the suitability of the habitat for competitors (as in the R* criterion of Tilman, 1982). Consequently, native lady beetles may have abandoned alfalfa fields to forage and reproduce elsewhere. Support for this habitat shift/compression hypothesis comes from field experiments in which native ladybirds "reappeared" in alfalfa fields in response to artificially created, local aphid outbreaks (Evans, 2004). As also consistent with the basic hypothesis, Kajita & Evans (2009a) determined that *C. septempunctata* presently succeeds to a much greater degree than does the native *C. transversoguttata* in reproducing in alfalfa fields with low aphid numbers. Analyses of survey data from diverse habitats throughout North America have enabled additional evaluation of the hypothesis as applied to *C. septempunctata* and other invasive ladybirds (Acorn, 2008; Finlayson *et al.*, 2008; Hesler & Kieckhefer, 2008; Gardiner *et al.*, 2009), but a clear consensus has not yet emerged.

Interspecific hybridization

Another hypothesis to account for declining populations of native ladybirds is that *C. septempunctata* might be hybridizing with them, as observed with other invasive species (e.g., Rhymer & Simberloff, 1996; Perry *et al.*, 2001; Snyder & Evans, 2006). Evidence to date for such is negative, however. Both sexes of *C. septempunctata* mated with *C. transversoguttata* when paired in the laboratory, but the eggs laid from such unions were not fertile (EWE, personal observations).

Additional studies are needed to determine further the extent and ecological basis of displacement of North American ladybirds by *C. septempunctata*. The example of *C. novemnotata* illustrates the challenges of unraveling what has happened (Wheeler & Hoebeke, 1995; Harmon *et al.*, 2007). Long-term censuses of alfalfa fields reveal that this native North American species was already rare in Utah even before *C. septempunctata* arrived in the early 1990s, and there is little indication that *C. novemnotata* has become rarer as *C. septempunctata* has become widely abundant (EWE, personal observations). Perhaps in contrast to events in northeastern North America (Wheeler & Hoebeke, 1995), buffering as provided by extensive native habitat in Utah has muted the adverse effects on this species from the establishment of *C. septempunctata* (e.g., see Evans, 2000, 2004). The more recent arrival of *H. axyridis* will now complicate the challenge of documenting and measuring non-target effects of *C. septempunctata*. It also raises the unwelcome possibility of a one-two punch from these two invasive ladybirds to the rare *C. novemnotata* and other North American species of concern such as *C. transversogutatta*, *A. bipunctata* and *L. melissa*. Long-term studies should continue, such that the ultimate outcomes for non-target species can be understood well in the wake of the establishment of hugely successful, invasive ladybirds in North America.

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Consequences of hybridization between invasive and biocontrol individuals of the harlequin ladybird *Harmonia axyridis* in France

Benoît Facon[1,*], Laurent Crespin[2], Anne Loiseau[1], Eric Lombaert[3], Alexandra Magro[4, 5] & Arnaud Estoup[1]

[1]INRA-SPE, UMR Centre de Biologie et de Gestion des Populations, Campus International de Baillarguet, CS 30016, 34988 Montferrier-sur-Lez cedex, France; [2]Evolutionary Ecology Research Group, Department of Biology - University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium; [3]UMR IBSV, INRA-UNSA-CNRS, 400 route des Chappes, 06903 Sophia-Antipolis Cedex, France; [4]Université de Toulouse; ENFA, EDB (Laboratoire Evolution et Diversité Biologique); 2 route de Narbonne, F-31320 Castanet Tolosan, France; [5]CNRS, EDB (Laboratoire Evolution et Diversité Biologique); F-31062 Toulouse, France; [*]Corresponding author: facon@supagro.inra.fr

Hybridization (interbreeding between genetically differentiated lineages) takes place in a very wide range of organisms (e.g. Mallet, 2005) and may play an active role in a variety of evolutionary processes ranging from local adaptation to speciation (e.g. Rieseberg *et al.*, 2003). In the field of invasion biology, hybridization is now seen as a potential stimulus for the evolution of invasiveness (Ellstrand & Schierenbeck, 2000; Lavergne & Molofsky; 2007; Blair & Hufbauer, 2009). Hybridization has the potential to boost invasiveness through two non-exclusive mechanisms: (i) heterosis (or hybrid vigour) if hybridization masks deleterious alleles (Keller & Waller, 2002) or in case of overdominance and/or synergistic epistasis between alleles inherited from the parental taxa. Allopolyploidy, which sometimes accompanies hybridization, may also contribute to the heterotic effect (Ainouche *et al.*, 2009); (ii) the generation of new genotypes through recombination (Ellstrand & Schierenbeck, 2000; Facon *et al.,* 2005), which alleviate the loss of genetic variance after founder events and hence restore if not increase the efficiency of selection (Lee, 2002).

To date, most studies dealing with admixture have aimed at detecting multiple source populations in biological invasions from selectively neutral markers (e.g. Kolbe et al., 2004). Only a few studies have explicitly investigated the consequences of such hybridization for the evolution of life-history traits and thus for the adaptative potential of the introduced populations (but see Lavergne & Molofsky, 2007; Wolfe *et al*., 2007; Facon *et al*., 2008). The purpose of the present study was to investigate the potential effects of a particular type of intraspecific hybridization in the ladybird *Harmonia axyridis* (HA) on the invasive process at work in Belgium and France since 2001 (Adriaens *et al*., 2003): the hybridization between the flightless biocontrol strain Coccibelle® (Tourniaire *et al*., 2000) and invasive individuals. As a matter of fact, Coccibelle® is still commercialized and one cannot exclude the possibility of crosses between this biocontrol strain and invasive individuals in Europe, especially in France. It is worth noting that such crosses would yield individuals able to fly and hence could potentially impact the invasive process.

According to Wolfe *et al*. (2007), three criteria must be met in order for intraspecific crosses to play a role in biological invasions. First, the populations involved in the admixture process should be genetically differentiated. Second, crosses should be possible between individuals from the different populations. Third, the admixed individuals should differ from parental ones in some of their life-history traits so as to impact the invasive process. In a recent study (Facon *et al*., 2010), we checked out the three above criteria in the case of the interaction between individuals from the flightless biocontrol strain Coccibelle® and from the invasive French population of HA. First, we evaluated the level of differentiation between Coccibelle® and the invasive French populations at 18 microsatellite markers (Loiseau *et al.*, 2009). Second, we studied reproductive barriers preventing interbreeding between biocontrol and invasive populations using a mate choice experiment. Third, we used a quantitative genetics experiment to estimate the phenotypic mean and variance for several life-history traits of offspring produced by intra- and inter-crosses involving the Coccibelle® and the French invasive populations.

We found that admixture between individuals from the French invasive population and from the flightless biocontrol strain of the harlequin ladybird can occur, and that, in agreement with the three criteria proposed by Wolfe *et al.* (2007), the invasive process can potentially be affected by such admixture. First we found that the two studied populations showed substantial genetic differentiation (F_{ST}=0.13). Second, crosses turned out to be possible between the involved populations, at least in laboratory conditions. Our mating experiment, based on trios of one female and two males (one of each population), clearly illustrates that no reproductive barrier has evolved between these two distinct HA populations as every crosses yielded viable offspring in similar proportions. Moreover, we found that males from the biocontrol flightless strain sired more offspring whatever the type of female. This result suggests that the cross between wild females and males from the biocontrol flightless strain might even be favoured *in natura*.

Third, the admixed individuals should differ from the parental ones in their life-history traits. Because pure individuals from the HA biocontrol flightless strain are unable to reach the wintering sites and thus to durably settle a sustainable population *in natura* due to their flightless phenotype, we focused our analysis on the comparison between pure invasive and admixed individuals. Interestingly enough, we found that invasive individuals never outperformed the admixed ones. This result highlights that the use of flightless individuals as biocontrol agents in the field could potentially enhance the invasive process by decreasing the Allee effect typical of dispersing individuals founding new populations (Tobin *et al.*, 2007). Indeed, in the invasive front, we could expect that population sizes are low. If recurrent releases of flightless individuals are made near the invasive front, they could thus decrease the Allee effect. A second important point is that we found that admixture had a significant impact on several studied traits. First, admixture impacted development time and adult body length, two traits for which we detected a significant shift in mean values. Hybrids (i.e. admixed individuals) displayed shorter development time and larger size than invasive individuals. These shifts in mean values are likely to correspond to a heterosis effect. Second, admixture increased the genetic variance for survival in starvation, with the genetic coefficient of variation (CV_G), of hybrids significantly exceeding parental ones for this trait. It is worth noting that for survival in starvation, there is no significant shift in the mean value for the admixed individuals but CV_G of the admixed crosses clearly exceeded parental ones, with some hybrid genotypes consistently outperforming parental ones. This result means that admixture increased the genetic variance for this trait and could boost the efficiency of selection in direction of higher survival under stressful conditions of starvation.

How can our results on development time, body length and survival in starvation could affect the invasive process of HA? The observed increase of body size in admixed individuals has the potential to impact the interactions between this species and the native coccinellid species by enhancing the dominance of HA in interspecific competition and intraguild predation (Polis *et al.*, 1989; Lucas *et al.*, 1998). It is worth noting that this increase in adult size does not occur at the expense of a longer development time. On the contrary, admixed individuals grow faster than invasive ones. This shorter development time should enhance population growth rate and hence impact the invasive potential of the species. Finally the third trait impacted by admixture is linked to survival in stress conditions (absence of food). Several studies pointed up that invasiveness may be associated with a higher stress-tolerance (e.g. Milne & Abbott, 2000). For HA, a longer survival in starvation conditions may be especially advantageous during periods of low occurrence of preys or in areas where preys are found at low density.

The three traits for which admixture had a positive effect are hence likely to be advantageous in an invasive context. Therefore, natural selection is expected to promote the introgression of genes from the biocontrol flightless strain in the wild and enhance the invasive potential of HA. It is worth stressing, however, that our results are only based on a F_1-hybrid generation. Additional studies over further generations are hence needed to forecast the long-term consequences of this hybridization event.

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Monitoring *Harmonia axyridis* intraguild predation on native coccinellids in the field

Louis Hautier[1], Jean-Claude Grégoire[2], Pierre Callier[3], Gilles San Martin [3,4], Jean-Pierre Jansen[1] & Jean-Christophe de Biseau[3]

[1]Département Lutte biologique et Ressources phytogénétiques, Centre wallon de Recherches agronomiques, Rue de Liroux, 2, B-5030 Gembloux, Belgique; [2]Lutte biologique et Ecologie spatiale, Université Libre de Bruxelles, CP 160/12, Av. F.D. Roosevelt 50 - 1050 Bruxelles, Belgique; [3]Eco-Ethologie évolutive, Université Libre de Bruxelles, CP 160/12, Av. F.D. Roosevelt 50 - 1050 Bruxelles, Belgique; [4]Present address: Écologie comportementale et conservation de la biodiversité , BDIV, Université catholique de Louvain, Croix du Sud 4 B-1348 Louvain-la-Neuve, Belgique;
Corresponding author: hautier@cra.wallonie.be

Less than ten years after its introduction for the biological control of aphids, the Multicoloured Asian Ladybird, *Harmonia axyridis* Pallas has invaded the whole of Belgium, including urban, agricultural and semi-natural habitats and has overlapped the niches of several native species (Adriaens *et al.*, 2008). In parallel, a decline of native ladybird species such as *Adalia bipunctata* (L.) and *A. decempunctata* (L.) was observed in tree habitats in Brussels (San Martin *et al.,* in prep). The causes of this decline are not clearly identified and could be connected to competition with or to intraguild predation (IGP) by *H. axyridis.* To track predation on native coccinellids in natural conditions, we developed a new method for detecting IGP by gas chromatography – mass spectrometry via prey exogenous alkaloids (Hautier *et al.*, 2008; Sloggett *et al.*, 2009). Using this method, IGP by *H. axyridis* in lime trees (*Tilia* spp.) was studied at twenty sites in Brussels in June-July 2008. Tree branches were beaten with a stick above a collecting net. *H. axyridis* larvae were isolated in microtubes and kept in a freezer until alkaloid analysis. Thirteen species of adult ladybirds were collected: in order of abundance, *H. axyridis, A. decempunctata*, *Calvia quatuordecimguttata* (L.), *C. decemguttata* (L.), *Halyzia sedecimguttata* (L.), *A. bipunctata*, *Propylea quatuordecimpunctata* (L.), *Exochomus quadripustulatus* (L.), *Oenopia conglobata* (L.), *Anatis ocellata* (L.), *Myrrha octodecimguttata* (L.) and *Aphidecta obliterata* (L.). Five species were caught as larvae: in order of abundance, *H. axyridis, C. quatuordecimguttata*, *Adalia* spp., *C. decemguttata* and *P. quatuordecimpunctata.* Thus *H. axyridis* was the most abundant species; with adults being present at all twenty sites and larvae at eighteen sites. An analysis of 590 *H. axyridis* larvae revealed the presence of exogenous alkaloids in 21% of the collected larvae and at nineteen sites. Positive larvae contained mainly one single alkaloid but in 6% of the positive larvae, two alkaloids were detected in each individual, resulting from double predation on two different coccinellid genera. Overall, three exogenous alkaloids were identified in *H. axyridis* larvae: adaline, propyleine and calvine. They are naturally present, respectively in *Adalia* spp., in *P. quatuordecimpunctata*, and in *Calvia* spp. (Laurent *et al.*, 2005). In conclusions, *H. axyridis* is becoming the dominant coccinellid species on lime trees in Brussels, both in terms of presence/absence at the sites and in terms of abundance. The analysis of the exogenous alkaloid content of *H. axyridis* larvae reveals the existence of IGP on native coccinellids. These results indicate that IGP is not a rare event in the aphidophagous guild and support the hypothesis that IGP by *H. axyridis* on *Adalia* spp. could explain the observed decline of the latter species in arboreal habitats.

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Predation behaviour of *Harmonia axyridis* on *Adalia bipunctata*

Louis Hautier[1], Etienne Branquart[2], Jean-Pierre Jansen[1] & Jean-Claude Grégoire[3]
[1]Département Lutte biologique et Ressources phytogénétiques, Centre wallon de Recherches agronomiques, Rue de Liroux, 2, B-5030 Gembloux, Belgium; [2]Belgian Biodiversity Platform (SPO), Centre de Recherche de la Nature, des Forets et du Bois (DGRNE), Avenue Marechal Juin, 23, B-5030 Gembloux, Belgium; [3]Lutte biologique et Ecologie spatiale, Université Libre de Bruxelles, CP 160/12, Av. F.D. Roosevelt 50 – 1050, Brussels, Belgium.
Corresponding author: hautier@cra.wallonie.be

Several years after the *Harmonia axyridis* invasion in Europe, a decline of some native species was observed. This decline could be due to the Asian ladybird, which is known as an intraguild predator of coccinellids (Cottrell & Yeargan, 1998; Snyder *et al.*, 2004; Ware & Majerus, 2008). In order to assess the incidence of intraguild predation by *H. axyridis* on the native species, *Adalia bipunctata*, experiments were run in Petri dishes without extraguild prey and on whole plants with extraguild prey. The predation behavior of one starved 4th instar of *H. axyridis* larva on an *Adalia bipunctata* egg batch or on one 1st, 2nd, 3rd or 4th instar larva was observed during 30 min and described according to the ethogram of Yasuda *et al.* (2001). For each combination, 20 replicates were observed and prey mortality was also recorded after 30 min and 24 h. The reverse interactions, the predation of *A. bipunctata* on *H. axyridis*, were observed as well. Experiments were repeated on caged aphid-infested broad bean plants. Six treatments with 2nd instar larvae were done and repeated five times: 5 *A. bipunctata* + 5 *H. axyridis*; 5 or 10 *A. bipunctata*; 5 or 10 *H. axyridis*; no larvae (aphid control). The cages were opened when pupation started and the numbers of larvae and aphids were recorded. In Petri dishes, *A. bipunctata* eggs and larvae were easily attacked by *H. axyridis* larvae, which displayed an aggressive behaviour towards all *A. bipunctata* instars. After 30 min, 80% mortality was observed among the three firsts instars larva. By contrast, the first larval instars of *H. axyridis* were less attacked by the fourth instar of *A. bipunctata*, supposedly because they were physically and/or chemically protected. Regarding the eggs, no significant differences of predation behavior were observed between the two species. After 24 h, all eggs of each species were completely consumed, which shows that their respective chemical defences were not effective. On caged plants, intraguild predation by *H. axyridis* on *A. bipunctata* was confirmed even in the presence of aphids and led to a decrease of the cannibalism in *H. axyridis* when the aphids became scarce. In conclusion, the eggs and larvae of *A. bipunctata* are potential intraguild prey for *H. axyridis* larvae. A different prey attack and predation behaviour of *A. bipunctata* was observed towards *H. axyridis* larvae but not towards eggs. The aggressive behaviour of *H. axyridis* and, possibly, its chemical or physical defences lead to an assymetric predation towards *A. bipunctata*. On plants with extraguild prey, intraguild predation by *H. axyridis* on *A. bipunctata* was confirmed and decreased cannibalism in *H. axyridis*.

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Harmonia axyridis as an economic pest of wine grapes in the U.S.: Progress in developing an IPM program and potential impact in Europe

William D. Hutchison, Tederson L. Galvan, Eric C. Burkness & Robert L. Koch
Department of Entomology, University of Minnesota, 219 Hodson Hall, 1980 Folwell Ave., St. Paul, Minnesota 55108 US.
Corresponding author: hutch002@umn.edu

Introduction

Since 2000, the multicolored Asian lady beetle (MALB), *Harmonia axyridis* (Pallas), has become an economically significant, contaminant pest of the wine making process throughout the eastern U.S. and eastern Canada. The beetles aggregate on clusters near harvest, and subsequently some beetles may be incorporated with the grapes during wine processing. Once crushed, the beetles, via 'reflex-bleeding,' release their foul smelling hemolymph that can taint the flavor and aroma of the resulting wine (Pickering *et al*., 2004; Galvan *et al*., 2007a). The "lady beetle taint" has primarily been attributed to several methoxypyrazines, known to occur in *H. axyridis* (Pickering *et al*., 2004).

Although MALB provides beneficial biological control of several aphid species in U.S. crops (e.g., pecan and soybean), the beetle's propensity to attack wine grapes is one of the most infamous non-target impacts of an introduced biological control agent (Koch and Galvan 2008). In most North American temperate climates, MALB dispersal to wine grapes (and apple, raspberry plantings) coincides with the senescence of nearby field crops (e.g., corn, soybean), where beetle populations had previously increased to high densities (Galvan *et al*., 2009a). We also have evidence that the late-season feeding on high-sucrose fruit (late Aug.-Sept.) may increase overwintering survival of MALB (Galvan *et al*., 2008).

Wine grapes in the Midwestern U.S. (e.g., Minnesota, Wisconsin, Iowa, Illinois) have a raw product value (prior to wine processing) of ca. U.S. $1,600 to 2,850 per ha (Galvan *et al*., 2006). Early in the MALB invasion and spread to North American grapes (e.g., 2003), some growers had to destroy several cases of wine, resulting in million dollar losses. Following the invasion of MALB in Minnesota in 1994, and subsequent infestations in wine grapes (particularly since 2000), we began working with several grape and wine producers to develop a comprehensive Integrated Pest Management (IPM) program (Galvan et al., 2009; Radcliffe *et al*., 2009) for growers in the Midwestern U.S., which is outlined below (see 5 steps). Although these elements of the IPM program are critical to successful management of MALB, research continues in the U.S. and Canada to better understand the MALB contamination process, as well as alternative methods for minimizing the risk of MALB "taint" to wine.

Since 2003, we have conducted research at several Midwestern U.S. vineyards to better understand the timing of infestations relative to harvest dates, and development of a practical Integrated Pest Management (IPM) program. While implementing the IPM program in Minnesota and Wisconsin, over a 3-year period (2004-2006), we found that 78% of the vineyards sampled had cluster infestations, near harvest, that were >10% (i.e., clusters with one or more beetles per cluster). We also confirmed that when infestations were >10%, one insecticidal spray near harvest provided a return of ca. $495/ha vs. the expensive alternative of using shaker tables and/or water/floatation systems to remove beetles after harvest. This return is based only on the raw product value of grapes, and is therefore a conservative estimate (see Galvan *et al.*, 2006a). With additional monitoring and insecticidal tools for managing MALB in the U.S., some regions have reported fewer problems with MALB. Table 1 provides a summary of a recent 2009 survey of entomologists and grape consultants in 5 major grape growing regions.

Table 1. Current pest status of the Multicolored Asian lady beetle (MALB), *Harmonia axyridis,* in the northern U.S. and eastern Canada, by five entomologists and grape consultants working with the wine grape industry, August, 2009 (1-5 scale)*

Survey results of grape entomologists: status of MALB (Nothern US and Canada)

Questions	Mean Response[1]
MALB remains a significant pest, wine grapes in your state/province?	3.8
Growers have a useful IPM-MALB plan?	3.2
A need for more effective IPM, MALB?	3.6
Growers, if necessary, will use chemical control (e.g. pyrethroids)?	3.8
Growers prefer non-chemical alternatives for control?	4.0

[1] 5=Strongly Agree; 4=Agree; 3=Neutral; 2=Disagree; 1=Strongly Disagree.

*Reflects ca. 10,250 ha of grapes in the North-Central, Northeastern States; Ontario, Can.

Review of MALB dynamics and IPM program for *Harmonia axyridis* in wine grapes

Timing of infestation

In the northern U.S. and Canada, MALB adult beetles begin to move to grape clusters between 2 and 3 weeks before harvest (Figure 1). This is the key "management window" for growers to be aware of for timely IPM. Even though growers can track how the population increases or decreases throughout the season, they should not worry about the beetles until the end of August (northern hemisphere), for varieties that will be harvested from early to mid-Sept. (Galvan *et al.*, 2006a-c). MALB are often found in vineyards as early as June when they are feeding on aphids or other small soft-body insects. Growers and crop advisors can follow the beetle increase in vineyards using yellow sticky traps, which can be used as an early warning tool as well (Figure 1).

Protection of grape berries

Laboratory and field studies have verified that MALB do not cause the direct, primary injury to grape berries (Koch *et al.*, 2004, Galvan *et al.*, 2006b). Fortunately, with all varieties tested thus far in the U.S., the beetle's mouth parts are unable to break the berry skin. Field sampling has shown the MALB population density increases as the incidence of berry injury, due to other causes, increases (Galvan *et al.*, 2006b). Therefore, the protection of berries by avoiding splitting (physiological/water stress) or bird damage, for example, will likely decrease the chances of having MALB in the clusters, even if beetles are in the vineyard. Wine grape varieties with tight clusters, such as 'Leon Millot,' may have a higher percentage of splitting, and will usually attract more lady beetles.

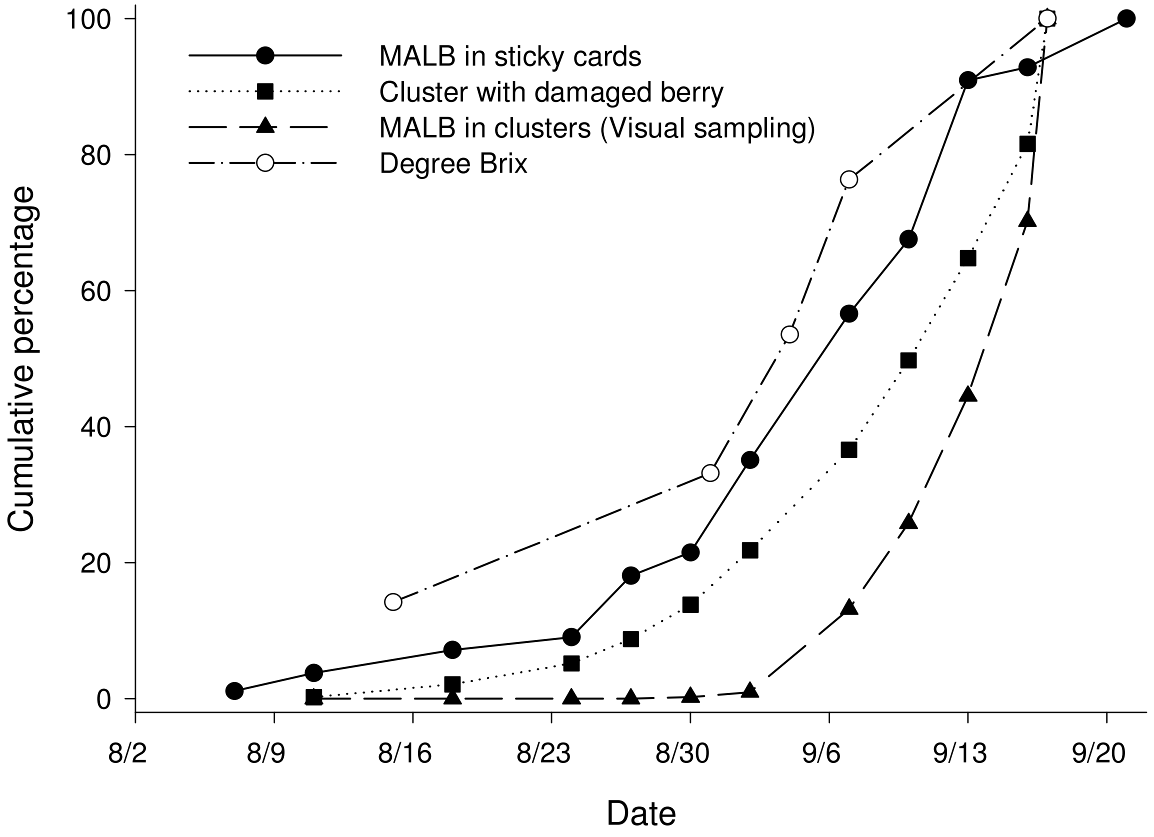

Fig. 1. Phenology of Multicolored Asian Lady Beetle (MALB) infestation levels and incidence of cluster damage and Brix percentage, near Hastings, Minnesota, 2004.

Scouting or monitoring

MALB monitoring should focus (or can be limited to) the 3-4 weeks prior to harvest, as noted with the management window (Figure 1). To sample for MALB adults, growers should randomly inspect 20 to 30 clusters per 3-ha vineyard, and record the percentage of clusters infested, with one or more MALB per cluster (see: Galvan *et al.*, 2007a,b). If the vineyard is greater than 3 ha, we recommend dividing the vineyard into two or more areas of no more than 3 ha each. Scouting can be done once or twice a week depending on the level of MALB infestation, and how close the grapes are to harvest. Growers can also estimate the infestation level by checking yellow sticky cards if those are available. This information can provide a relative estimate of whether the population is increasing or decreasing as harvest approaches. As the grapes get closer to harvest, consider sampling more frequently, every other day, to avoid any unpleasant surprises on the day of harvest. MALB populations may increase quickly within a few days.

Making a decision

The numbers of lady beetles per cluster that should trigger a control action, such a spraying an insecticide, depend on consumer preference, grape variety, and the grower. Consumers have different responses to off-flavors in wine, some are tolerant and some are sensitive. Grape and wine chemistry varies with grape varieties and wine styles, and some of the chemicals found in wine may mask or accentuate the off-flavor caused by MALB. Finally, some grape growers or wine makers are riskier than others, and would allow a higher level of MALB in their vineyard than the less risky grower. For example, a study done in our lab in collaboration with the Sensory Center from the University of Minnesota found that 1 MALB per 4 clusters (or 25% of clusters infested) of 'Frontenac' would give enough off-flavor to be noticed by 10 % of wine consumers (Galvan *et al.*, 2007a). A grower who is not a risk taker probably would want to reduce this percentage, and accept only 1 MALB per every 10 clusters (or 10% of clusters infested). Therefore, after sampling 30 clusters, and finding 3 clusters infested with 1 or more MALB, one should make a decision to control (e.g., insecticide spraying) the lady beetle population in that vineyard. As a rule of thumb, if one is unsure what the best threshold would be for your situation, we would recommend a conservative threshold of 10% of the clusters infested (Galvan *et al.*, 2007b).

Control options

Management of MALB can and should be done by protecting the grape berries from any type of injury. For example varieties that are less sensitive to physiological splitting (due to rapid increase in water uptake), or drip irrigation to better manage water use, should be considered. Insecticides should only be used if MALB populations reach high levels (e.g., 10% of clusters infested with lady beetles; Galvan *et al.*, 2007b). Likewise, mechanical removal of MALB from clusters during harvest by floating clusters in water, or vacuuming clusters should be reserved for high MALB populations. If growers in the U.S. decide to use an insecticide, our laboratory and field studies have shown that 4 products work reasonably well for control of MALB in wine grapes (Galvan *et al.*, 2006c), including: carbaryl applied at 7 days before harvest, or pre-harvest interval (PHI), and imidacloprid (Provado™) or the pyrethroids, cyfluthrin (Baythroid™) and (Mustang-Max™) all with a 1-day PHI in the U.S. (Galvan *et al.*, 2006c). Importantly, we are only aware of these products being registered for grapes in the U.S.; we are not yet aware of similar control options in Europe. Also, one critical limitation to the use of carbaryl is that although a high percentage of beetles will be killed soon after application, many beetles will remain in the cluster after dying; thus, depending upon the harvest date, these beetles may still contain enough compounds to taint the wine. Final insecticide choice should be based on the time between the insecticide application and projected harvest (PHI), MALB infestation levels, and cost and safety to field workers. As always, growers should follow the directions on the label when applying insecticides. Some insecticides may be restricted use and may require a pesticide applicators license to purchase product. Finally, we also evaluated the impact of all insecticides on important wine quality parameters (brix content, acid content at harvest) and wine taste; we did not find negative effects on these parameters.

Caveat and Adaptation of IPM Recommendations

The IPM program and recommendations for managing MALB are based on research conducted in the Midwestern U.S. from 2004 to 2009. Other locations with different levels of MALB infestations, or unique climatic or agroecosystem conditions that affect the phenology and dispersal of MALB may require site-specific IPM programs. Also, if a given grape variety is known to be more sensitive to the *Harmonia* taint, or if multiple flights of MALB occur, then treatment levels should be lower. For example, a more conservative treatment threshold of 3-5 % of clusters infested, near harvest, may be necessary for some regions.

Future IPM strategies

Current research in Minnesota includes the development and evaluation of a “push-pull” system for potential control of MALB in wine grapes. Several compounds have been identified as attractants (to ‘pull’ beetles out of grapes) and repellents (to ‘push’ beetles out); thus far we have found that beta-caryophyllene may have potential as an effective attractant under field conditions (ECB, TLG, unpublished data). However, much more work needs to be done to verify the most consistent repellent and attractant compounds, as well as the overall cost and feasibility of the method for commercial growers. This approach, if successful, would allow growers to avoid or minimize late-season insecticide use near harvest.

Possible impacts in European grapes

A recent assessment of the thermal requirements for population growth of MALB in tandem with a climate matching analysis (Poutsma *et al.*, 2008), found that most of western Europe, including several grape production regions in France, Germany and Switzerland, were highly conducive to beetle establishment. Since 2004, the continued spread of MALB in Europe further supports continued establishment of the beetle in several grape growing regions. Interestingly, a recent report from Switzerland documented increases in light trap catches of MALB in one grape growing area (Linder, 2009). Although no MALB were found on grape clusters in Switzerland in 2009, significant infestations were found in one region in Germany. Given our experience with MALB in North American grapes, we anticipate that the following factors may continue to predispose much of the wine-grape industry in Europe to significant infestation risks, including:

- Temperate climates, that provide conditions favorable for overwintering, and assist to induce a diapause phase (e.g., cooling to sub-freezing ambient temperatures in Sept-October), that appears to be important for fruit, “sugar” feeding during autumn, prior to overwintering
- Wine grape production regions that are near significant hectares of field crops that harbor large aphid populations (e.g. maize), allowing MALB to build up by late summer
- Grape varieties that are increasing in sugar content (Brix) in late August to Sept., as nearby field crops are senescing
- Grape varieties that are sensitive to physiological splitting, incur bird damage, or damage from pathogens, all of which cause premature damage to grape berry skin, allowing MALB to feed within grapes and grape clusters

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Intraguild predation between *Harmonia axyridis* and the hoverfly *Episyrphus balteatus*

Brecht Ingels & Patrick De Clercq
Ghent University, Department of Crop Protection, Laboratory of Agrozoology, Coupure Links 653, B-9000 Ghent, Belgium.
Corresponding author: Brecht.Ingels@Ugent.be

Intraguild predation (IGP) occurs when two predators that share the same prey also consume each other. It is a widespread interaction among insect natural enemies, that is believed to have a strong influence on the structure of communities (Polis *et al.,* 1989; Pell *et al.,* 2008). The invasive harlequin ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) is a well known intraguild predator in interactions with other predatory Coccinellidae (Pell *et al.,* 2008). However, little is known about its interactions with other predators of the aphidophagous guild, like hoverflies. *Episyrphus balteatus* (De Geer) (Diptera: Syrphidae) is one of the most common hoverfly species in Europe, and its larvae are voracious aphid predators in different crops (Hindayana *et al.,* 2001). The species is commercially available in Europe for the biological control of aphid pests. In the present study, we have investigated the occurrence of IGP between *H. axyridis* and *E. balteatus* in Petri dish arenas with a leaf disk of broad bean (*Vicia faba*) on agar. The interactions were examined between larvae, pupae and adults of *H. axyridis* and larvae and pupae of *E. balteatus*, both in the absence and presence of the pea aphid *Acyrthosiphon pisum* (a mixture of late instars and adults) as extraguild prey.

IGP between *H. axyridis* and *E. balteatus* was found to be asymmetric, with the coccinellid in the majority of cases being the intraguild predator and the syrphid the intraguild prey. In the absence of extraguild prey, only third instars of *E. balteatus* acted on a number of occasions as intraguild predator on first instars of *H. axyridis*. Also in the absence of extraguild prey, first instars of *E. balteatus* were most sensitive to IGP, as they were killed by all stages of *H. axyridis*. Second instars and third instars of the syrphid were only killed by older larvae and adults of *H. axyridis.* Pupae of either species were not attacked.

In the presence of extraguild prey, the frequency of IGP was substantially reduced in most of the combinations tested. However, IGP still occurred, mainly in combinations of older larvae of *H. axyridis* with first or second instars of *E. balteatus*. The declines in the frequency of IGP were the strongest in combinations with third instars of *E. balteatus*. Possibly, in the absence of extraguild prey, the syrphid larvae were weakened because of a lack of food during the experiment. As such, they were not able to effectively defend themselves against the older coccinellid larvae. In contrast, well fed syrphid larvae appeared to be strong enough to defend themselves. The syrphid larvae tried to shake off their attacker by producing slime secretions, and by fiercely thrashing their body.

In conclusion, *H. axyridis* appears to be a strong intraguild predator in interactions with the hoverfly *E. balteatus* in Petri dish arenas. The presence of extraguild prey reduces the frequency of IGP significantly, but does not eliminate its occurrence. Further research using plant arenas will be conducted to get a more realistic view on the interactions between these predators. Also, IGP events between these predators in the field will be assessed using molecular methods.

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Intraguild predation between *Harmonia axyridis* and European ladybirds: do egg surface chemicals provide protection in some native species?

Angelos Katsanis, Marc Kenis & Dirk Babendreier
CABI Europe–Switzerland, Rue des Grillons 1, 2800 Delémont, Switzerland
Corresponding author: a.katsanis@cabi.org

Harmonia axyridis is an Asian ladybird that is presently invading Europe (Brown *et al.*, 2008). This species is suspected to affect native populations through intraguild predation and competition for food. It is known to feed on eggs of many coccinellid species both in field and laboratory conditions (Ware & Majerus, 2008). Chemical protection of eggs against predators is a common feature in insects (Marples, 1993, Hemptinne *et al.*, 2000a, Blum & Hilker, 2002, Sato & Dixon, 2004). In the framework of a project studying intraguild predation between *H. axyridis* and native ladybirds, we investigated egg predation by young larvae and potential chemical protection mechanisms. We focused our observations on intraguild interactions between *H. axyridis* and native aphidophagous coccinellids that share the same resource and are thus likely to encounter the invasive species in the field.

Four experiments were carried out. In the first experiment, we tested the palatability of eggs of ladybird species by *H. axyridis* larvae, and vice-versa. One individual first instar larva and one egg were introduced simultaneously in a 3cm diameter Petri dishes. Egg predation was observed during 24 hours. The experiments were replicated 30 times. The following native ladybird species were tested: *Adalia bipunctata*, *Adalia decempunctata*, *Anatis ocellata*, *Aphidecta obliterata*, *Calvia decemguttata*, *Calvia quatuordecimguttata*, *Coccinella septempunctata*, *Harmonia axyridis*, *Hippodamia undecimnotata*, *Hippodamia variegata*, *Oenopia conglobata* and *Propylea quatuordecimpunctata*. The tests showed that the eggs of most indigenous species are more palatable to predation by *H. axyridis* larvae than eggs of *H. axyridis* are to predation by larvae of the indigenous species, except for eggs of *Calvia quatuordecimguttata*, which were not eaten by *H. axyridis* larvae, as observed in an earlier study by Ware *et al*. (2008). In contrast, eggs of the congeneric species *Calvia decemguttata* were heavily preyed by *H. axyridis*. In addition, all *H. axyridis* eggs were preyed by their own larvae.

In order to assess whether the observed resistance to predation in *C. quatuordecimguttata* eggs was due to egg surface chemicals, another experiment was conducted in similar conditions. One individual *H. axyridis* larva was introduced with an egg that had been previously washed in n-Hexane for 2 minutes to remove egg surface chemical compounds (Hemptinne *et al*., 2002, Ware *et al*., 2008). The species used for this experiment were *C. decemguttata, C. quatuordecimguttata* and *H. axyridis*. Egg predation levels on *C. decemguttata* and *H. axyridis* did not change significantly when these eggs were washed with n-Hexane. In contrast, the eggs of *C. quatuordecimguttata* clearly became more acceptable to predation by neonate *H. axyridis* larvae after washing.

To confirm whether the chemical compounds that are found on the surface of the eggs affect the palatability of indigenous ladybird eggs by *H. axyridis* larvae, we washed eggs of the same three species with n-hexane and then re-applied their own extract on their surface. The level of palatability of these eggs was similar to the original unwashed eggs of the same species. Finally, we washed coccinellid eggs from the same three species and then applied on their surface the extract of the egg of another species. These tests showed that *C. quatuordecimguttata* eggs became more vulnerable to predation by *H. axyridis* larvae , when treated with the extracts of the *C. decemguttata* and *H. axyridis* eggs. On the contrary, *C. decemguttata* and *H. axyridis* eggs, when treated with the extract of *C. quatuordecimguttata* eggs, became more vulnerable to *H. axyridis* larvae. These experiments clearly showed that chemical compounds that are found on the surface of these eggs are responsible for the difference in palatability by *H. axyridis*. Chemical analyses of the eggs will be conducted to identify the chemicals involved in the protection of the eggs.

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Impact of *Harmonia axyridis* on European ladybirds: which species are most at risk?

Marc Kenis[1], Tim Adriaens[2], Peter Brown[3], Angelos Katsanis[1], Johan Van Vlaenderen[1], Rene Eschen[1], Laurence Golaz[1], Renate Zindel[1], Gilles San Martin y Gomez[4], Dirk Babendreier[1] & Remy Ware[5]
[1]CABI Europe-Switzerland, Rue des Grillons 2, 2800 Delemont, Switzerland;.[2]Research Institute for Nature and Forest, Kliniekstraat 25, B-1070 Brussels; [3]Department of Life Sciences, Anglia Ruskin University, East Road, Cambridge, CB1 1PT, UK; [4]Catholic University of Louvain-la-Neuve, Ecology and Biogeography Unit, Croix du Sud, 4-5 (Carnoy), B-1348 Louvain-la-Neuve; [5]Department of Genetics, University of Cambridge, Downing Street, Cambridge, CB2 3EH, UK
Corresponding author: m.kenis@cabi.org

Introduction

Since the introduction of the harlequin ladybird, *Harmonia axyridis* (Pallas), in Europe, and its establishment in more than 15 countries (Brown *et al*., 2008), there has been increasing concern regarding its potential impact on indigenous ladybirds. Both laboratory and field experiments have shown that *H. axyridis* is a voracious intraguild predator that can feed on eggs and larvae of most ladybirds and compete effectively for resources (Koch & Galvan, 2008; Pell *et al*., 2008; Ware & Majerus, 2008). Field studies are presently being carried out in various countries in Europe to monitor changes in populations of native ladybird species following the arrival of *H. axyridis* (e.g. Eschen *et al.*, 2007; Adriaens *et al.*, 2008; Tomov *et al*., 2010). However, ladybird populations are known to fluctuate naturally and, thus, it will take several years of monitoring before a long-term impact can be demonstrated. Hence, we decided to carry out a risk assessment for the individual native ladybird species in Europe, to identify those species that are most and least at risk following the invasion of *H. axyridis*.

The risk posed by *H. axyridis*, on native ladybirds can be considered as the product of the likelihood that a given ladybird species encounters *H. axyridis* in the field, and the consequence of this encounter, through direct predation and competition for food (van Lenteren *et al*., 2008). Therefore, the risk assessment was carried out in three steps: (1) likelihood of encounter with *H. axyridis* in the field; (2) consequence of competition; (3) consequence of predation.

Likelihood of encounter

Ladybird larvae and adults were collected in various habitats and ecosystems recently invaded by *H. axyridis* in Switzerland. To assess which species most often encounter *H. axyridis* in the field, an asymmetric co-occurrence index was calculated for the most common native ladybird, defined as the percentage of specimens of a given species collected at the same time and the same site as *H. axyridis*. Data from Switzerland were merged with similar recent indexes from Belgium (Adriaens *et al.,* 2008) and a general niche overlap index (1-3) was developed and provided for the 30 most common native ladybirds in Europe. Since the method is based on calculations of niche overlap, it is only applicable for an already established non-native species. However, the capacity of a species to cause damage outside of its native range is most often believed to be the single most reliable predictor of impact (cf. Williamson, 1999).

In Switzerland and Belgium, *H. axyridis* was found in nearly all habitats surveyed. However, niche overlap was greater with arboreal aphidophagous species found on broadleaved trees than with ladybirds found mainly or exclusively in grasslands or on conifers. European species that will most likely encounter *H. axyridis* during their lifetime are *Adalia bipunctata* (L.), *Adalia decempunctata* (L.), *Calvia quatuordecimguttata* (L.), *Calvia decemguttata* (L.) and *Oenopia conglobata* (L).

Consequence of competition

The consequence of competition for food between *H. axyridis* and a given European species was assigned to a numerical scale (1-3), based on the degree of dietary overlap between them, as described in the literature (Majerus, 1994; Hodek, 1996; Klausnitzer & Klausnitzer 1997). Species that were principally aphidophagous were considered most at risk from competition for food (score 3); species feeding principally on non-aphid homopterans were considered at moderate risk (score 2); species utilising more unusual diets, such as mildew and plant matter, were considered the least at risk (score 1). Among the 30 most common native species, 19 were scored “3” and five “2”.

Consequence of predation

The consequence of intraguild predation was assessed in laboratory studies. Various competition tests between *H. axyridis* and 14 indigenous species were carried out. The impact of predation by *H. axyridis* was also applied to a quantitative scale (1-3), which took into account any chemical, physical and behavioural defensive strategies. A score “3” was assigned to species for which all life stages are highly susceptible to predation by *H. axyridis* and for which behavioural avoidance is unlikely (e.g. *Adalia* spp.). A score “2” was given to species which are susceptible to predation, but in which behavioural avoidance is known (e.g. *Coccinella septempunctata* L.). A score “1” was given to species for which physical or chemical defences are present at all or some life stages (e.g. *Anatis ocellata* (L.) and *C. quatuordecimguttata*). For species where no laboratory data could be obtained, we made informed extrapolations based on field observations and published information. If no information was available, a species was scored “2”.

Final risk assessment

A total risk index score was calculated for each native species, by multiplying the score of the likelihood of encounter, by the sum of the two consequence scores. Four species obtained the maximum score of 18: *A. bipunctata, A. decempunctata*, *C. decemguttata* and *O. conglobata*. It remains to be seen whether the long-term observations of field populations will confirm the decline of these particular species. In contrast, herbivorous and mycetophagous species are much less at risk because they do not compete for resources and are less likely to encounter *H. axyridis* in the field.

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Can IGP be responsible for the success of *Harmonia axyridis*?

Pavel Kindlmann
Department of Theoretical Ecology, Institute of Systems Biology and Ecology AS CR, Na sádkách 7, CZ-370 05 České Budějovice, Czech Republic and Institute of Environmental Studies, Charles University, Benátská 2, CZ-12801 Prague 2, Czech Republic.
Email: pavel.kindlmann@centrum.cz

Abstract: There is no doubt that *Harmonia axyridis* has been successful in suppressing many native species in North America and subsequently in Europe. It is not quite clear, however, what are the ecological mechanisms that enabled it to be so successful – is it higher fecundity, more generations per year, or competitive superiority, which enable it to suppress native species? Here I critically examine, whether its' competitive superiority, lending it an advantage in intraguild interactions, can be an important factor. Empirical data show that the incidence of intraguild predation (IGP) between ladybird species seems to be very low under natural conditions. A simple population dynamics model, however, shows that despite this, IGP might play a substantial role in the success of *H. axyridis* under some circumstances.

Key words: *Harmonia axyridis*, intraguild predation

Introduction

Intraguild predation (IGP) has become a major research topic in biological control and conservation ecology. It occurs when two predator species compete for the same prey and one of them also feeds upon its competitor (Polis *et al.*, 1989) and is assumed to be a widespread interaction within many, but not all, communities of biological-control agents (Rosenheim *et al.,* 1995; Holt & Polis, 1997). As a consequence, IGP combines two important structuring forces in ecological communities: competition and predation (Polis & Holt, 1992; Polis & Winemiller, 1996), and may generate a diversity of indirect effects among co-occurring species (Miller & Brodeur, 2002).

In a recent paper, Kindlmann & Houdková (2006) used a simple model developed for the simulation of population dynamics of aphidophagous insects, which was based on the type of egg distribution made by predatory females, to estimate the real strength of IGP in the aphidophagous insects. The model was used to estimate the proportion of predatory individuals that face a conflict with a heterospecific competitor at least once during their life. Based on this, predictions were made on the population dynamics of both predatory species. The predictions were confronted with real data on IGP in ladybirds. Both the theoretical model and the empirical data supported the hypothesis that direct interspecific conflicts between aphidophagous predators seem to be rather infrequent in the field conditions. Here I show that that despite this, IGP might play a substantial role in the success of *H. axyridis* under some circumstances.

Model assumptions

We consider the dynamics of two ladybird species, A and B. We assume that:

1. The incidence of encounters between individuals of A and B is very low, in accord with Kindlmann & Houdková (2006), and therefore also the probability of IGP, i.e., of cases when larvae of A and B meet and compete (and presumably one consumes the other), is very low.
2. Larval mortality of both A and B is very large [consistent with Osawa (1993) and Kindlmann *et al.* (2000)].
3. Larvae of species A are competitively superior to those of species B in mutual contests (if they meet and fight).

Results and discussion

The model can be expressed in general terms; here I give only one demonstrative numerical example. Assume we have 10000 aphid colonies and each of the two species (A and B) reproduces at randomly chosen 100 colonies (i.e., in $\frac{1}{100}$ of the colonies) and leaves 10 eggs in each of these colonies. Due to the randomness of oviposition of both species, only 1 of the colonies will contain offspring of both species, as $\frac{1}{100} \times \frac{1}{100} = \frac{1}{10000}$. Thus there will be 99 colonies, each containing 10 eggs of A, 99 colonies, each containing 10 eggs of B, and 1 colony containing 10 eggs of A and 10 eggs of B.

Assume 99% larval mortality (Osawa 1993). Then, out of the 99 colonies containing eggs of species A, we expect only 9.9 = .01*99*10 survivors - adults of species A. Similarly, we expect approximately 9.9 survivors of species B out of the 99 colonies containing eggs of species B. In the single colony containing both species the survivor – if any – will belong to species A because of its competitive superiority. However, because of the assumed 99% larval mortality, there is only a 10% chance that there will be any survivor in this colony at all.

Putting this all together, we expect 10 survivors of species A and 9.9 survivors of species B, i.e., the ratio of fitnesses of both species is A:B = 10:9.9 = 1.01. Thus, despite of rarity of IGP (which is absent in $\frac{9999}{10000}$ aphid colonies in our simplified example), the ratio of fitnesses of both species is $\frac{100}{99}$, and therefore not negligible.

The model can be framed in general terms with the same result, and will be published in full elsewhere. Its' take-home message will, however, remain unchanged, if juvenile mortality of two species (one IG predator and one IG prey) is very large, then even a very low incidence of encounters between these two species may lead to a relatively large ratio between their fitnesses in favour of the IG predator and therefore ultimately to replacement or strong suppression of IG prey by IG predator. Thus, IGP might play a substantial role in the success of *H. axyridis* under some circumstances.

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Harmonia axyridis: six-legged alligator or lethal fugu?

Oldřich Nedvěd [1,2], Plamen Kalushkov [3], Xenia Fois [4], Dita Ungerová [1] & Adéla Rozsypalová[1]
[1]Faculty of Sciences, University of South Bohemia, and [2]Institute of Entomology, Biology Center, Academy of Sciences of the Czech Republic; both Branišovská 31, 370 05 České Budějovice, Czech Republic; [3]Institue of Zoology, Bulgarian Academy of Sciences, 1 Tsar Osvoboditel blvd. Sofia 1000, Bulgaria; [4]Istituto per lo Studio degli Ecosistemi CNR, sede di Sassari, Italy
Corresponding author: nedved@prf.jcu.cz

The phenomenon of intraguild predation (IGP) among aphidophagous ladybirds attracted large attention and has been a subject of a considerable number of studies (*e.g.* Pell *et al.,* 2008; Ware & Majerus, 2008; Sloggett & Davis, 2010), especially after recent invasion of the Multicoloured Asian ladybird *Harmonia axyridis* in Europe (Brown *et al.,* 2008). IGP is presented as an important factor contributing to the success of *H. axyridis*. Less attention has been paid to interactions of *H. axyridis* with other intraguild (=aphidophagous) organisms (e.g. entomopathogenic fungi, Baverstock *et al.,* 2009; or hoverflies, Alhmedi *et al.,* 2010). Studies on toxicity and unpalatability of ladybirds to potential non-IG predators, usually ants and birds (Pasteels, 1973, 2007, Burgio *et al.*, 2008, Dolenská *et al.,* 2009) may contribute to the explanation of the invasive success of *H. axyridis*.

Aggressive invasive and large ladybird *H. axyridis* was previously proved as a powerful intraguild predator winning combats with most other ladybirds, except large *Anatis ocellata* (Ware & Majerus, 2008). We have investigated the occurrence of IGP between larvae of *H. axyridis* and several other aphidophagous insects and non-aphidophagous ladybirds in Petri dish arenas. One larva of each of two tested species was placed into 9cm Petri dishes at 20°C and 18L:6D photoperiod, with wet filter paper on the bottom. Survival of the larvae was recorded after 24 hours.

Larvae of the third instar of the lacewing *Chrysoperla carnea* (Neuroptera, Chrysopidae) won in 94% of cases over the third instar ladybird of the same size. When the third instar larvae of the lacewing were paired with the fourth instar ladybird larvae, which were three times heavier, lacewings still won in 52% of cases, the ladybird won in 24% cases, and both larvae survived 48 hours in another 24%.

Larvae of the hoverflies (Diptera: Syrphidae) were partially protected (27% for *Episyrphus balteatus*) or strongly protected (76% for *Epistrophe eligans*) against intraguild predation but they never killed *H. axyridis* larva of similar size. Slime prevented the syrphid larvae from being killed for long time, suggesting that protection from IGP by *H. axyridis* is even higher under natural conditions. In similar experiments, Ingels & De Clercq (2010) found that only third instars of *E. balteatus* acted as intraguild predators on first instars of *H. axyridis*, and only in the absence of extraguild prey. Second and third instars of the syrphid were only killed by older larvae and adults of *H. axyridis*, while pupae were not attacked (Ingels & De Clercq, 2010). In laboratory microcosms containing aphids on plants, *H. axyridis* larvae had an IGP advantage over the hoverfly (Alhmedy *et al.* 2010).

We also paired third instar larvae of *H. axyridis* with fourth instar spiny larvae of the phytophagous ladybird *Cynegetis impunctata* (being of similar size). *H. axyridis* won in 67% of cases, while both survived in 33% of cases. Second instar larvae of *H. axyridis* killed fourth instar larvae of the ladybird *Scymnus rubromaculatus* (again of similar size) in all nine cases studied, despite their defensive waxy structures.

We tested toxicity of whole body extract from several species of ladybirds on young individuals of the water flea, *Daphnia magna* (Cladocera), because they cannot avoid the toxins. Ladybirds of known mass were homogenized in water, centrifuged, and clear supernatant was diluted to set of concentrations. Toxicity of *Coccinella septempunctata* for *D. magna* was low: 4mg of fresh body mass per ml of water were needed to kill 50% of water fleas after 24 hours at 20°C, while *H. axyridis* was very toxic: lethal dose was 0.06mg/ml. Diverse colour forms and developmental stages of *H. axyridis* showed similar toxicity. Toxicity of *Adalia bipunctata* was intermediate, 0.6 mg/ml was needed to kill 50% of water fleas.

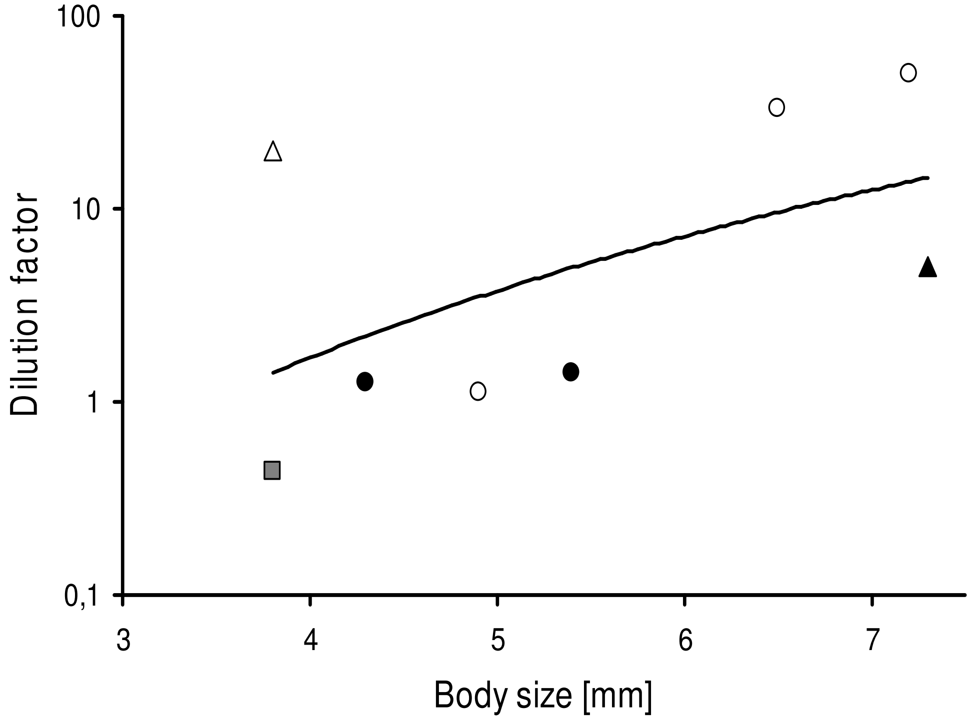

Fig. 1: Relationship between dilution factor (how many times diluted body liquid of ladybird is still repellent to 50% of ant individuals) and ladybird body size. Circles=aphidophagous species, triangles: mycophagous species, square: phytophagous species. Open symbols: bright yellow or red background with black spots, solid symbols: dark background with lighter spots, grey symbol: brown, without spots.

We studied unpalatability of extracts from eight ladybird species mixed with sucrose solution for the ant *Lasius niger*. Whole body extracts were diluted to a set of concentrations provided that sucrose concentration was constantly 4%. We provided one droplet of extract and one droplet of pure sucrose solution each time to a laboratory stock of ants, and counted the number of ants drinking from either droplet within 3 minutes. According to the reaction of ants to diverse concentrations, we calculated the dilution factor (how many times diluted body liquid of ladybird is still repellent to 50% of ant individuals). The ladybirds were ranked according to the dilution factor: *Coccinella septempunctata* (50x) >*Harmonia axyridis* (33x)

>*Psyllobora vigintiduopunctata* (20x) >*Halyzia sedecimguttata* (5x) >*Calvia quatuordecimguttata* (1.4x) >*Exochomus quadripustulatus* (1.3x) >*Adalia bipunctata* (1.1x) >*Cynegetis impunctata* (0.4). Reaction of ants was slightly more rejective to the extracts of larger ladybirds (Fig. 1) and of more aposematically coloured species (bright backgroung with black spots). Mycophagous species were rejected similarly as the aphidophagous ladybirds.

We here report on the first aphidophagous insect (*C. carnea*) which is superior intraguild predator over *H. axyridis* despite its smaller size. However, in the majority of IGP trials we conducted, *H. axyridis* was found to be superior to competitors, supporting the general picture. *H. axyridis* larvae could overcome spines, wax, and to some extent slime. In addition, our results on high toxicity and repellence of *H. axyridis* to other arthropods including its potential predators indicate an ecological advantage of *H. axyridis* compared to other ladybirds.

Acknowledgements

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Establishment of *Harmonia axyridis* in the Netherlands: successful aphid control and/or ecological disaster?

C. Lidwien Raak-van den Berg, Peter W. de Jong & Joop C. van Lenteren
Laboratory of Entomology, Wageningen University, PO box 8031, 6700 EH, Wageningen, The Netherlands.
Corresponding author: lidwien.raak-vandenberg@wur.nl

Introduction

The Multicoloured Asian Ladybird (*Harmonia axyridis*), indigenous in China and Japan, has recently invaded northern America and Europe. Its area in Europe is still expanding. Little is known about its biological characteristics in the Dutch/European natural environment. The aim of our project is to study several of the characteristics of *H. axyridis* in the Netherlands in order to gain insight into the causes and consequences of their rapid establishment. Research themes and first results of our work on *H. axyridis* in the Netherlands are presented below.

Life-history and population biology-cohort analysis

What are the life history characteristics of *H. axyridis* in natural and semi-natural environments in Western Europe and how do they differ from those of native ladybird species? In the summer of 2009, 3 cohorts of larvae of both *H. axyridis* and *Adalia bipunctata* had been released on caged, espalier formed, lime trees (*Tilia europaea* 'Pallida') infested with *Eucallipterus tiliae*. Life tables will be constructed based on the data obtained.

Preliminary results indicate that the developmental times for the indigenous *A. bipunctata* and the exotic *H. axyridis* are similar. Larval and pupal survival of *H. axyridis*, however, are much higher than survival of *A. bipunctata.*

Diapause or quiescence

Does *H. axyridis* show diapause? In 2008 hibernating adults were collected and placed in overwintering sites behind a window shutter. During the winter of 2008-2009, every two weeks 30 pairs were transferred to 25°C and 16:8 L:D conditions. First we determined whether females started laying eggs. If they did, the pre-oviposition period was recorded.

In total about 13.500 adults were collected. Three specimens of form *axyridis* and 2 of f. *equicolor* were found. Specimens of the latter form had black dots on the red front half of the elytra. The preliminary results suggest that *H. axyridis* does not show a real diapause. Individuals taken from the overwintering cages start to lay eggs shortly after being transferred to 25°C and long-day conditions (ranging from 8-13 days at the beginning of December to 4-6 days at the end of March).

Interactions with other ladybirds

How does *H. axyridis* interact with indigenous ladybirds? Is there any recognition of presence of other species, e.g. based on other semiochemicals? Individual behavioural observations are made to answer these questions.

Phenology and distribution

What is the phenology and distribution of *H. axyridis* in the natural environment in the Netherlands? In spring and early summer of 2009, ladybird numbers were counted at several set locations around Wageningen, the Netherlands. Preliminary results suggest that *H. axyridis* is mostly found in urban environments. In more natural situations the aphid densities as well as ladybird densities are much lower.

Winter survival

What is *H. axyridis'* survival during winter in the Netherlands*?* Overwintering survival in natural and semi-natural situations will be studied during the winter of 2009-2010.

Literature study

Known data on life history characteristics of *H. axyridis* are reviewed. The biology of *H. axyridis* in the Netherlands and Western Europe will be compared with its biology in the native range (China and Japan) to get a better understanding of what keeps their numbers low in Asia. Ultimately we intend to compare *H. axyridis'* biology with the biology of another exotic coccinellid used as a biological control agent in the past, but which apparently has not been able to establish itself in the Netherlands yet: *Hippodamia convergens.*

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Assessing the potential use of *Coccipolipus hippodamiae,* a sexually transmitted ectoparasite, as a control agent of invasive populations of the ladybird *Harmonia axyridis*

Emma L. Rhule*, Michael E. N. Majerus, Francis M. Jiggins & Remy L. Ware
Department of Genetics, Downing Street, Cambridge, CB2 3EH, U.K.
**Corresponding author: e.rhule@gen.cam.ac.uk*

Abstract: Invasive populations of *Harmonia axyridis* pose a threat native biodiversity. The potential use of the sexually transmitted ectoparasite, *Coccipolipus hippodamiae*, has been investigated as a method of population suppression, filling the role of natural enemies in the native range. Here it is shown that *C. hippodamiae* is able to establish, reproduce and induce female sterility in a novel host. Additionally, host switching by *C. hippodamiae* from known hosts to *H. axyridis* has been recorded in wild Polish populations. Further research must be conducted in order to accurately assess whether *C. hippodamiae* should be released as a bio-control agent of British *H. axyridis* populations.

Key words: *Harmonia axyridis; Coccipolipus hippodamiae;* sexually transmitted disease; biological control

Introduction

The enemy release hypothesis is often invoked to explain the invasion success of species occurring outside their native range (Torchin *et al.,* 2003, Colautti *et al*., 2004, Carroll *et* al., 2005). This may be due to a lack of natural population control usually exerted by sympatric predators, parasites and pathogens. This is not due to the lack of natural enemies, but rather that enemies in the introduced range will only have co-evolved adaptations to host species with which they have been sympatric for a large part of their evolutionary history, and not necessarily with the invasive species. The natural or artificial introduction of natural enemies into populations of invasive species can therefore be an effective control method.

The ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) is native to Asia, but, in recent decades has been introduced into Europe and North America as a biological control agent of aphids and coccids (Brown *et al*., 2008a), where it has rapidly spread and had a detrimental impact on native species. This is attributable not only to its ability to outcompete native ladybirds and other aphidophages for food (Brown and Miller, 1998, Colunga-Garcia and Gage, 1998), but because, being a polyphagous predator, *H. axyridis* will also attack non-pest insects (including lepidopteran eggs and larvae (Koch *et al*., 2003)), and other aphidophages (including the immature stages of other coccinellids) (Pell *et al*., 2008, Ware and Majerus, 2008, Ware *et al*., 2008).

A promising candidate for the biological control of this species is the sexually transmitted ectoparasitic mite *Coccipolipus hippodamiae* (McDaniel & Morrill) (Acarina: Podapolipidae). Naturally occurring in some European coccinellid populations including *Adalia bipunctata* (Linnaeus) and the congeneric *Harmonia quadripunctata* (Pontoppidan)*,* incidence of the mite can reach a high prevalence and induce host sterility (Hurst *et al*., 1995, Webberley *et al*., 2004). In order for *C. hippodamiae* to successfully invade and persist within a given population, there must be high levels of promiscuity to permit horizontal transmission between host individuals, and overlapping generations to facilitate transmission between successive generations. *Harmonia axyridis* in Britain is highly promiscuous, (Majerus and Roy, 2005), long-lived, and undergoes at least two overlapping generations each year (Brown *et al*., 2008b). Therefore, it is possible that *C. hippodamiae* may be able to spread through populations of *H. axyridis.*

Material and methods

To ascertain whether *C. hippodamiae* could be introduced into populations of a novel host, wild collected samples of three species, *A. bipunctata*, *H. quadripunctata* and *H. axyridis* were collected and scored for *C. hippodamiae* infection, as per Webberley *et al*., 2004. Mite-infected *A. bipunctata* and *H. quadripunctata* individuals provided a source of mites for transfer to uninfected individuals. Artificial transfer followed the protocol of Hurst *et al.* (1995). The recipient ladybirds were scored for mite presence, and numbers of adults, eggs and larvae noted. *Adalia bipunctata* individuals were infected to provide a control for the *H. axyridis* transfers. Successful establishment was defined as complement of mite development on the new host resulting in at least one sexually reproducing adult female with eggs present.

Infected individuals were then maintained in Petri dishes with uninfected individuals to determine whether or not *C. hippodamiae* could undergo sexual transfer in a novel host. This ability was compared with that in *A. bipunctata.*

The egg hatch ratios of two stocks of female *H. axyridis*, one infected the other uninfected, and both fed a daily diet of excess pea aphids were observed to monitor fertility. Females were mated once a fortnight and egg clutches were collected every other day. Petri dishes were changed daily. Again, stocks of *A. bipunctata* provided control data.

In May and June 2009, samples of *H. axyridis* were collected in and around Torún and Bydgoszcz, Poland, by eye and by beating trees. The *C. hippodamiae* infection status of collected individuals was scored; those infected were preserved in 100% ethanol.

Further details of the materials and methods used during this research can be found in Rhule et al., 2009 (in press).

Results and discussion

We report that colonies consisting of reproducing adult mites and their eggs can be established on the novel host *H. axyridis* via artificial transfer from, and sexual contact with, infected individuals from known host species. Once infected, *H. axyridis* individuals can then infect conspecifics during copulation. Infection induces sterility in females. The long-term effects on males are not yet known.

In addition, we report the natural occurrence of *C. hippodamiae* in recently established Polish populations of *H. axyridis* demonstrating that the abilities of the mite to transmit and reproduce on *H. axyridis* are not just phenomena restricted to a laboratory context. However, the field prevalence of the mite infected individuals is currently low. Investigations into the mating rate, the dynamics of mite transmission, the effect of mite infection on host survival and the degree of overlap between generations will determine whether *C. hippodamiae* ultimately reaches a high prevalence and has a significant impact on *H. axyridis* populations.

By definition, risk assessments must look to the future; progressive global warming increases the possibility that British coccinellids will become fully bivoltine, and as such, mites introduced into native British species through interspecific matings with *H. axyridis* could establish permanent infections. However, taking into account the fact that the mite is already present in Northern European populations of species commonly found in Britain and that sporadic infections occur in southern regions of the UK as a result of introductions by infected migrants, it is likely that the mite would colonize Britain anyway.

The question that must now be addressed is whether an artificial introduction should be implemented. Given the many negative effects resulting from the introduction of *H. axyridis* into continental Europe, and the subsequent invasion of the UK, a considerable degree of research is required before another species is released outside of its' native range.

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Changes in coccinellid fauna monitored by light traps

Hans Peter Ravn[1] & Jan Pedersen[2]
[1]Forest & Landscape, University of Copenhagen, Hørsholm Kongevej 11, DK-2970 Hørsholm; [2]Zoological Museum, Natural History Museum of Denmark, University of Copenhagen, Universitetsparken 15, DK-2100 Copenhagen
E-mail: hpr@life.ku.dk

Since 1992 a light trap has been collecting flying insects on the roof of Zoological Museum in Copenhagen. The trap is serviced with intervals ruled by flight activity of Lepidoptera. However, the catch of Coleopterans is identified as well. Not all coccinellid species are attracted to light, but 15 species of the 52 species recorded from Denmark have been caught more or less abundant. *Harmonia axyridis* is one species that have been caught in higher numbers since it was recorded for the first time: 12 specimens in 2006, 8 in 2007 and 229 in 2008 (see figure 1).

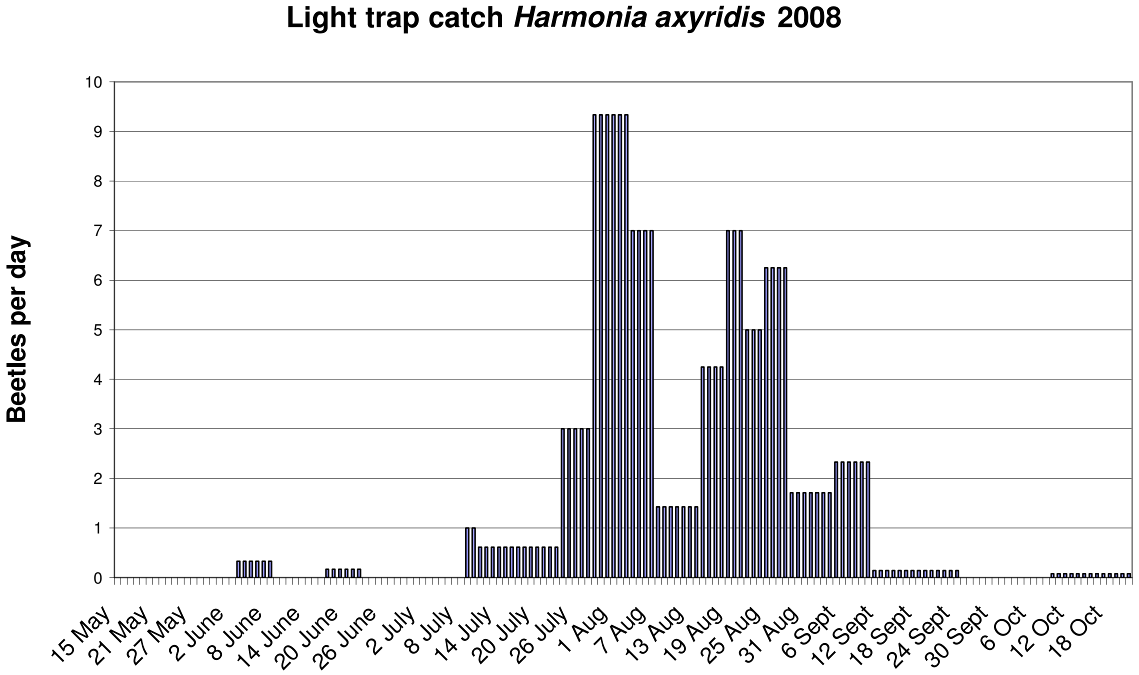

Figure 1: The catch reflects the flight activity and how this is influenced by the present weather conditions and abundance of the species.

Some of the native coccinellid species believed to be threatened by the invasion of the *H. axyridis* – e.g. *Adelia bipunctata* and *A. decempunctata* – are monitored as well. Changes in their general abundance could be monitored in a rather simple way. Preliminary results indicate this tendency for *A. bipunctata*.

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Ectoparasitic mite and fungus on *Harmonia axyridis*

Eric W. Riddick
National Biological Control Laboratory, Agricultural Research Service, USDA, Stoneville, Mississippi, 38776, USA.
Corresponding author: eric.riddick@ars.usda.gov

Ectoparasitic mites (Acarina: Podapolipidae) and ectoparasitic fungi (Laboulbeniales: Laboulbeniaceae) occur on ladybirds (Coleoptera: Coccinellidae) throughout the world (Riddick *et al.,* 2009). This study documents the interaction of a coccinellid-specific mite *Coccipolipus hippodamiae* (McDaniel & Morrill) and fungus *Hesperomyces virescens* Thaxter in *Harmonia axyridis* (Pallas) overwintering aggregations (Riddick, 2010). The objectives of this research were to (1) determine the prevalence of both parasites and (2) estimate the effect of parasitism on winter survival of the host. The prevalence of mite and fungus in host aggregations ranged from 3.2 to 17.4% and 3.6 to 43.5%, respectively, in winter aggregations in five different locations in Mississippi, southeastern USA, from 2007-2009. The mite colonized the subelytral space of male and female *H. axyridis*. Mite prevalence was greatest on fungus-infected beetles, especially females. Fungus prevalence was greatest on the abdomen of *H. axyridis* males that harbored mites than those that did not. *Harmonia axyridis* adults (particularly males) infected with the mite and fungus or the fungus alone had lower survival rates, under simulated winter conditions, than those not infected with any parasites. This research documents the parasitic mite *C. hippodamiae* infecting *H. axyridis* under natural field conditions for the first time anywhere in the world (Riddick, 2010). There is a need to identify effective methods of managing *H. axyridis* to diminish its negative impacts in some urban and agricultural landscapes worldwide (Kenis *et al.*, 2008). This suite of natural enemies could assist in controlling *H. axyridis* populations in targeted locations.

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Encounters with an alien: *Harmonia axyridis* in Europe

Helen E. Roy[1] & Peter M. J. Brown[2]
[1]Centre for Ecology & Hydrology, Benson Lane, Crowmarsh Gifford, Oxfordshire, OX10 8BB, UK; [2]Department of Life Sciences, Anglia Ruskin University, East Road, Cambridge, CB1 5PT, UK
Corresponding author: hele@ceh.ac.uk

The harlequin ladybird, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) is an aphidophagous coccinellid, native to central and eastern Asia (Kuznetsov, 1997; Majerus *et al*., 2006). It was introduced to Europe in 1982 and has spread rapidly since, for example, 100 km per year in Britain. *Harmonia axyridis* has been recorded as established in: France (first report), Germany, Belgium, Netherlands, Switzerland, Luxembourg, England, Czech Republic, Italy, Austria, Denmark, Norway, Poland, Wales, Liechtenstein, Scotland, Hungary, Slovakia and Bulgaria (last report). The IOBC/WPRS (International Organization for Biological Control/Western Palaearctic Regional Section) Study Group "Benefits and Risks of Exotic Biological Control Agents" was established to encourage collaborative research on this species and other exotic biological control agents. In this paper we describe some of the current activity in Europe.

Harmonia axyridis has been available in many countries for use as a biological control agent of pest insects including aphids and scale insects. Since 1916 *H. axyridis* has been repeatedly released as a classical biological control agent in the USA (Gordon, 1985; Majerus, 1994). It did not establish until the 1980s but then spread and increased in abundance so that it is now the most numerous of all ladybirds throughout North America (Hesler *et al.,* 2001; Smith *et al.,* 1996; Tedders and Schaefer, 1994). In Europe it has been commercially available for both classical and inundative biological control strategies since 1982 (Iperti and Bertand, 2001) and has become widely established (Brown *et al*., 2008a). The expansion of *H. axyridis* in Europe has been met with considerable concern both from ecological and anthropogenic perspectives (Roy and Wajnberg, 2008). It is widely considered an invasive alien species because it poses a threat to native biodiversity through competition and predation.

The spread of *Harmonia axyridis* has been spectacular, for example, it arrived in England in 2004 and has spread across Britain at approximately 100km per year in Britain (Brown *et al*., 2008b). The UK Ladybird Survey has been monitoring *H. axyridis* since it arrived in Britain through an online public participation survey: www.ladybird-survey.org. The survey has received more than 30,000 *H. axyridis* records. The pattern of rapid spread and high abundance of *H. axyridis* has also been documented across northern and central Europe (Brown *et al*., 2008a). *Harmonia axyridis* has been recorded as established in (order relates to approximate time of establishment): France (first report), Germany, Belgium, Netherlands, Switzerland, Luxembourg, England, Czech Republic, Italy, Austria, Denmark, Norway, Poland, Wales, Liechtenstein, Scotland, Hungary, Slovakia and Bulgaria (last report). It is interesting that this species is not so successful in southern, as opposed to northern, European countries (Soares *et al*., 2008).

Harmonia axyridis is both a human nuisance, in the autumn it occupies premises in high numbers, and also threatens native biodiversity through competition and predation. However, it is essential that the extent of the threat is quantified and this is a research area that needs urgent attention. There is no doubt that collaborative effort is essential to further understanding of this conspicuous invader and the IOBC/WPRS (International Organization for Biological Control/Western Palaearctic Regional Section) Study Group "Benefits and Risks of Exotic Biological Control Agents" was established to encourage collaborative research on this species and other exotic biological control agents.

The role of *H. axyridis* as an intraguild predator has been the focus of a many studies (Nóia *et al.*, 2008; Roy *et al.*, 2008a; Ware and Majerus, 2008; Ware *et al.*, 2008). *Harmonia axyridis* acts as an aggressive, unidirectional intraguild predator of many coccinellids (Ware and Majerus, 2008). In the 2008 special issue of *BioControl* (Roy and Wajnberg, 2008) the intraguild interactions between *H. axyridis* and other aphidophagous organisms were reviewed (Pell *et al.*, 2008). It is apparent that much of the evidence involves rigorously controlled laboratory experiments. The challenge now is to examine the role of *H. axyridis* as an intraguild predator in the field.

The natural enemy escape hypothesis is a theory that is used to explain the rapid (and uninterrupted) spread of an alien invader. The alien arrives in a region which represents natural enemy free space and so escapes the top-down regulation of predators, parasites and pathogens. The arrival of *H. axyridis* in Europe provides an opportunity to examine this concept. Coccinellids in Europe have many natural enemies; mainly parasites and pathogens (Hodek and Honěk, 2006; Kenis *et al.*, 2008). Experimental work has assessed the potential of two ladybird natural enemies as mortality agents of *H. axyridis*: the parasitoid wasp *D. coccinellae* (Koyama and Majerus, 2008) and the fungal pathogen *Beauveria bassiana* (Roy *et al.*, 2008b). In both studies, *H. axyridis* was less susceptible than native species of ladybird to the natural enemy. However, it is difficult to envisage that an insect at such high density will remain free of natural enemies within its invaded range; indeed populations of *H. axyridis* in Europe represent a large resource pool for natural enemies. Therefore, it is predicted that natural enemies, particularly parasites and pathogens, will begin to adapt to utilizing *H. axyridis*. Throughout 2009 the UK Ladybird Survey has received the first evidence that a low proportion of field-collected individuals of *H. axyridis* are host to parasites and pathogens in Britain. In Denmark, the focus has been on pathogenic fungi, and extensive field sampling has found larvae, pupae and adults to be infected by *Isaria farinosa*, *B. bassiana* and species of *Lecanicillium* (Steenberg and Harding, 2009).

In conclusion, *H. axyridis* is often reported as an invasive alien species with the potential to threaten native biodiversity and cause human nuisance (Majerus *et al.*, 2006). However, it is critical that empirical evidence, on the extent of these effects (particularly on species within the aphidophagous guild), is gathered to enable us to have a thorough understanding of this invasive alien species. Furthermore, we have a unique opportunity to monitor this alien across Europe, through public participation, and to study this species within a community context through extensive biological recording in the field. If we couple this with intensive and systematic field and laboratory studies, in the collaborative spirit of the IOBC Study Group, we will disentangle the dynamics of this invasive alien species.

Acknowledgements

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Sex wars and alien invaders

Helen E. Roy
NERC Centre for Ecology & Hydrology, Benson Lane, Crowmarsh Gifford, Oxfordshire, OX10 8BB, UK
Corresponding author: hele@ceh.ac.uk

Abstract: The susceptibility of *Adalia bipunctata*, infected with either *Wolbachia*, *Rickettsia* or *Spiroplasma*, to the fungal pathogen *Beauveria bassiana* was examined. Individuals infected with *Wolbachia* were no more susceptible to the fungal pathogen than uninfected (normal sex ratio) individuals. In contrast, ladybirds infected with either *Rickettsia* or *Spiroplasma* were more susceptible to *B. bassiana*. The evolutionary implications of these results are briefly discussed.

Key words: Male-killing bacteria, Coccinellidae, *Adalia bipunctata*

Introduction

Sex distortion in Coccinellids was first observed in *Adalia bipunctata* (Coleoptera: Coccinellidae) more than sixty years ago (Lusis, 1947). However, it was only relatively recently that Hurst *et al.* (1992) predicted that the causative agent was a cytoplasmically inherited bacterium and subsequently confirmed it to be the vertically transmitted bacterium *Wolbachia* (Hurst and Majerus, 1993). Two further bacteria (*Spiroplasma* and *Rickettsia*) have since been discovered to also cause sex distortion. These maternally transmitted endosymbionts are thought to infer fitness compensation; the females gain an advantage through consumption of their dead siblings.

Mike Majerus was particularly fascinated with sex ratio distortion and more specifically ultraselfish endosymbionts such as *Wolbachia*, *Spiroplasma* and *Rickettsia*). This paper is a tribute to Mike (an inspirational mentor) in which I tell the story of our collaborative work on the interactions between male-killers and the pathogenic fungus *Beauveria bassiana* (Ascomycota: Clavicipitaceae).

Methods

Adalia bipunctata adults were reared on pea aphids, *Acyrthosiphon pium* (Homoptera: Aphididae) in laboratory conditions (20°C +/- 2°C; 16 L:8 D). Four lines of *A. bipunctata* were maintained: one normal sex ratio (NSR) line (equal numbers of male and female progeny produced) and three abnormal sex ratio lines (*Wolbachia*, *Rickettsia* and *Spiroplasma*. In all the three abnormal sex ratio lines the presence of the specific bacterium was tested using molecular techniques and observation of distorted sex ratios in egg batches.

Adult ladybirds were dipped in 1ml of *B. bassiana* spore solution (10^7 spores per ml) or as a control, 0.03% Tween 80. Ten adults were dipped per treatment group: Male NSR, Female NSR, *Wolbachia*, *Rickettsia* and *Spiroplasma* (note in all the sex ratio lines the ladybirds were female). Each individual ladybird was placed in a separate container (5mm diameter single vented Petri dish) and maintained under controlled conditions (22°C; 16 L:8

D). High humidity was achieved for the first 24 hours by surrounding the dishes in paper moistened with sterile distilled water. The ladybirds were fed daily on an excess of mixed instar pea aphids. Mortality was monitored daily.

Results

There was no control mortality (ladybirds dipped in 0.03% Tween 80). There was mortality in all other treatment groups. Sixty percent of ladybirds from the *Rickettsia* and *Spiroplasma* sex ratio lines died from *B. bassiana* infection (Figure 1). In contrast only 40 percent of the *Wolbachia* sex ratio line died and this was a similar level of mortality to the NSR adults (males and females).

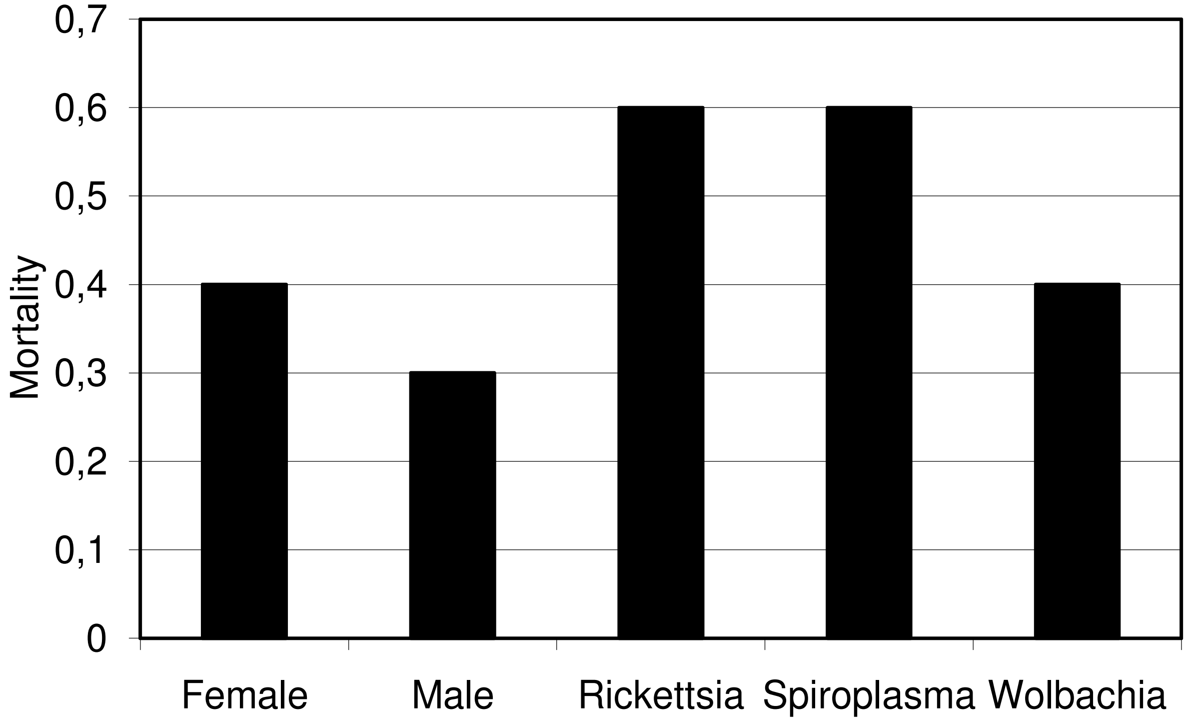

Figure 1: Proportion mortality of adult ladybirds (NSR: Female and Male, Sex ratio lines: *Rickettsia*, *Spiroplasma* and *Wolbachia*) dipped in *B. bassiana* 10^8 spores per ml.

Discussion

These preliminary results suggest that there is no cost to the *Wolbachia* carrying ladybirds in terms of susceptibility to the fungal pathogen *B. bassiana*. In contrast ladybirds carrying *Rickettsia* and *Spiroplasma* appear to be more susceptible to the fungal pathogen. There have been a number of recent papers examining the relationship between *Wolbachia* and viral infection of insects. Hedges *et al.* (2008) demonstrated that *Drosophila melanogaster* flies infected with *Wolbachia* are less susceptible to mortality induced by a range of RNA viruses. In our study ladybirds carrying *Wolbachia* infection did not incur an advantage or a disadvantage in terms of resistance or susceptibility to a fungal pathogen. In contrast, *Rickettsia* and *Spiroplasma* infected *A. bipunctata* appeared to be disadvantaged in that fungal induced mortality was higher compared to the NSR individuals. From an evolutionary perspective an endosymbiont is disadvantaged if it weakens its host and, therefore, *Wolbachia* would seem to have developed a more advanced strategy than either *Rickettsia* or *Spiroplasma*. However, if death of the host, through some mechanism, increased horizontal transmission of the sex distorting endosymbiont then a fitness advantaged could be accrued by weakening the host's resistance to infection. Understanding of these intriguing micro-organisms and their ecological interactions is at an early stage but further investigation will reveal the intricacies of these intimate relationships between host, endosymbiont and pathogen.

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Michael (Mike) E.N. Majerus was an inspirational mentor to many, many people. The work described here was collaborative research undertaken in the haven of Mike's field station. I am extremely fortunate to have had the opportunity to work alongside Mike and value our many discussions beyond actual words. It was a great privilege to convene a symposium in tribute to Mike and to celebrate the enthusiasm and knowledge fostered by Mike in so many of his ladybird friends.

Tribute to Professor Mike Majerus: an inspirational mentor.

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Invasive alien species in Europe: a review of the patterns, trends and impacts reported by the DAISIE project

David B. Roy
NERC Centre for Ecology & Hydrology, Maclean Building, Benson Lane, Crowmarsh Gifford, Wallingford, Oxfordshire, OX10 8B, UK
Corresponding author: dbr@ceh.ac.uk

Abstract: Early warning and prevention of the harmful impact of alien species on ecosystems is a fundamental requirement of the European Biodiversity Strategy and the EU Action Plan to 2010 and Beyond. In the absence of reliable regional analyses however, the European states have been unable to tackle this issue strategically. As part of the Europe response, a pan-European inventory of invasive alien species has been developed through the DAISIE (Delivering Alien Invasive Inventories for Europe) project. The DAISIE database has collated information on over 11,000 introduced species of fungi, plants, vertebrates, and invertebrates in Europe, and of more than 50,000 records of introductions. The rate of invasion for most species groups has become more rapid over the last century and clear differences in pathways of introduction have been identified. Most invasive plants and associated invasive invertebrates arrive in Europe via horticulture and agriculture, most vertebrates are deliberately release or escape from captivity and marine invasions are closely linked to shipping patterns. DAISIE has a key role within ongoing developments to tackle invasive alien species in Europe and is potentially a model for other continents which currently have much less detailed information on their alien biota.

Introduction

Effective control of invasive alien species has been hampered in Europe by the lack of (1) monitoring for alien species at frequent enough intervals in regions of concern; (2) a means to report, verify the identifications, and warn of new sightings; and (3) risk assessments that predict the likelihood of a particular species becoming invasive. Information on the invasive alien species present in Europe is incomplete, and that which is available is scattered in a variety of published and unpublished accounts and databases. Anticipating invasions by alien species is difficult, because access to information on their previous invasive ability (one of the best predictors of whether a new species will become invasive) is mostly unavailable. A key recommendation of the European Strategy on Invasive Alien Species is the development of a regional inventory of alien species recorded in the wild (Council of Europe, 2002).

In response to this requirement, the European Commission, launched the DAISIE project (2005-2008). The general objectives of the project were:

1. To create an inventory of alien species that threaten European terrestrial, fresh-water and marine environments
2. To structure the inventory to provide the basis for prevention and control of biological invasions through the understanding of the environmental, social, economic and other factors involved
3. To assess and summarise the ecological, economic and health risks and impacts of the most widespread and/or invasive species in Europe
4. To use distribution data and the experiences of the individual Member States as a framework for considering indicators for early warning.

By achieving these objectives, DAISIE aimed to deliver a European "one-stop-shop" for information on biological invasions in Europe.

Implementation

DAISIE focused on four major areas of information gathering and dissemination:

1. The European Alien Species Expertise Registry: a directory of researchers and research
2. European Alien Species Database: including all known alien species in Europe
3. European Invasive Alien Species Information System: descriptions of key alien species known to be invasive in Europe that includes distribution maps of key invasive alien species in Europe known or suspected of having environmental or economic impacts.

Each of these activities is briefly described below and they have been integrated together as a single internet portal for information on European alien species (www.europe-aliens.org), and supported by communication, data sharing and dissemination activities.

European Alien Species Expertise Registry

Current expertise in biological invasions is distributed across research organisations throughout Europe and is funded mainly by national programmes. In response, DAISIE established the European Alien Species Expertise Registry, representing a fundamental step towards linking these organisations and individuals in ways that provide added value at the European level and builds the critical mass of expertise in invasive alien species research to meet European-scale requirements. The registry contains information on the field of expertise (distribution, conservation, ecology, economy, genetics, legislation, management, pathways, physiology, risk assessment, and taxonomy) and on the taxonomic and geographic structure of the expertise. As of 2009, the Registry contains information on 1,737 experts from 92 countries for almost 3,400 higher taxa (family level or higher).

European Alien Species Database

An up-to-date inventory of all alien species known to inhabit Europe is essential to building an early detection and warning system for the Europe's environmental managers. This critical step represented the major activity in DAISIE and involved compiling and peer-reviewing national lists of hundreds of species of fungi, plants, invertebrates, fish, amphibians, reptiles, birds and mammals. As of 2009, the number of alien taxa known to be present somewhere within Europe is 11,494. The list represents a broad taxonomic spectrum of terrestrial and aquatic free living and parasitic organisms. The DAISIE database (www.europe-aliens.org) also contains documented introduction records of the alien taxa for 71 terrestrial and nine marine regions of Europe, the Levantine Basin and the North African coast of the Mediterranean Sea. The total number of species-region records is 50,001, including 46,173 records for terrestrial regions and 4,284 for aquatic regions (Table 1).

Besides the taxonomy and data on regional introduction records for each alien species the DAISIE database contains information on synonyms, ecofunctional grouping, species status (native in some parts of Europe but alien in other parts of Europe, alien to Europe, or cryptogenic, i.e. origin is unknown and it cannot be distinguished whether it is native or alien), introduction time, data on ecological and economic impacts, invasion history, population status as well as comments as free text. Sources of the data can be backtracked to original references and contributors.

Table 1. Numbers of species-region records representing different taxonomic/biome groups in the DAISIE database (as of 1st October 2008).

Taxonomic/biome group	*Number of introduction events*
Aquatic marine	2729
Aquatic inland	1555
Terrestrial invertebrates	12331
Terrestrial vertebrates	1373
Terrestrial plants	31300
Terrestrial fungi	1169

The DAISIE database is constantly growing due to increased knowledge, arrival of new alien species, spread into new regions (i.e. addition of new introduction records), changes in species taxonomy, etc. Therefore it is advisable to visit the DAISIE portal at www.europe-aliens.org for the most up-to-date information and use the database exploration tools presented therein.

European Invasive Alien Species Information System

One of the primary tools for raising awareness on biological invasions has been the publication of species accounts of the most prominent alien invaders. The species accounts realised within the DAISIE project have been prepared with the purpose of delivering a synthesis of the most relevant up-to-date information on the ecology, distribution and impact of 100 of the most invasive species. The DAISIE species accounts include three terrestrial fungi species, 18 terrestrial plant species, 16 terrestrial invertebrate species, 15 terrestrial vertebrate species, 16 species found in inland waters and 32 species from coastal waters. These species invade European natural and semi-natural habitats and already cause or have the potential to cause severe environmental, economic and/or health problems. The species were nominated to the list by experts working within the DAISIE research project. They are perhaps not the 100 most invasive alien species in Europe, but rather representatives of all main taxonomic groups and all environments and were selected so as to represent diverse impacts on ecology, socio-economic values and human and animal health. The species accounts were written by experts in the specific taxon and are based on the most up to date information available, both published and unpublished.

Conclusions

It is hoped that the inventory, accounts, and distribution maps will provide a qualified reference system on invasive alien species in Europe, available online for environmental managers, legislators, researchers, students and all concerned. The current inventory highlights that the rate of new naturalizations has consistently declined for some taxa most notably vertebrates such as inland fish, birds and mammals, while consistent increases are found for many invertebrates in both aquatic and terrestrial biomes (Figure 1). However, even where rates of establishment are declining, the cumulative number of alien taxa is increasing and for plants, marine invertebrates and terrestrial insects, current rates of increase are over 10 new taxa per year. The pan-European inventory of alien species created through DAISIE provides a platform for European reporting on biodiversity indicators and highlights areas where Europe will need to direct resources to manage biological invasions.

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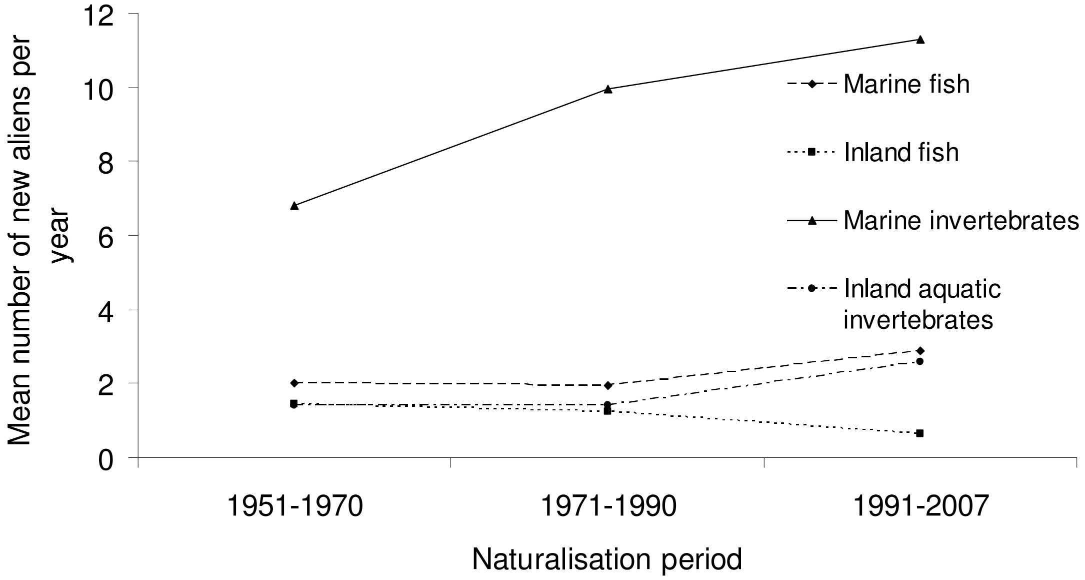

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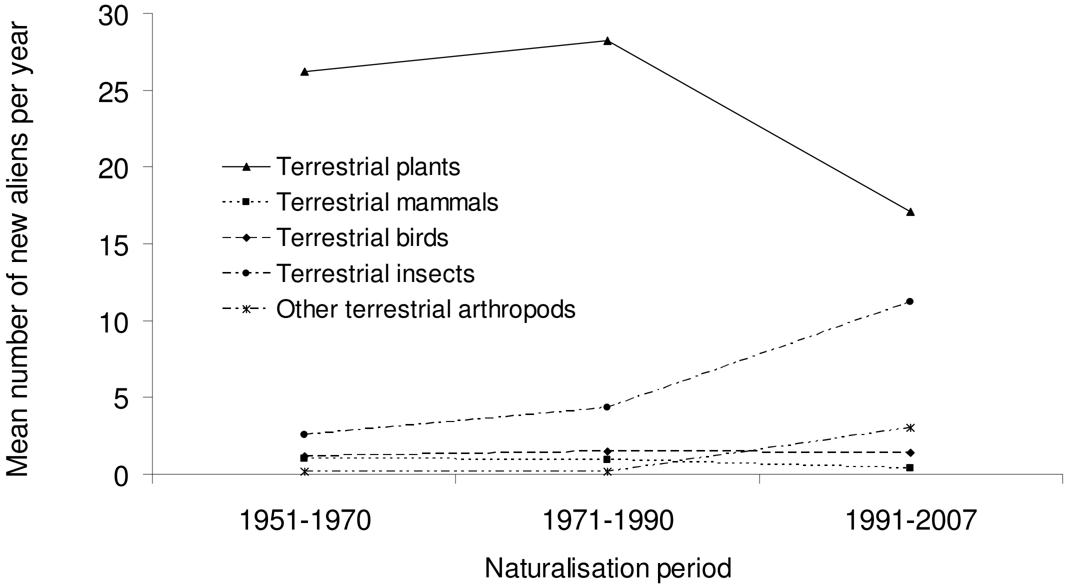

Figure 1. Pan-European trends in the average number of new alien plants, invertebrate, fish, birds and mammals naturalising in Europe per year in three time periods1951-1970; 1971-1990 and 1991-2007 in a) aquatic and b) terrestrial environments. From DAISIE (2009).

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The history of *Harmonia axyridis* (Pallas 1773) in Norway

May-Guri Sæthre, Arnstein Staverløkk & Trond Hofsvang
Bioforsk – Norwegian Institute for Agricultural and Environmental Research, Plant Health and Plant Protection Division, Department of Entomology and Nematology, Høgskoleveien 7, 1432 Ås, Norway.
Corresponding author: may-guri.saethre@bioforsk.no

Abstract: The history of *Harmonia axyridis* and a summary of surveillance and research in Norway till June 2009 are given. *H. axyridis* was assessed as a potential bio-control agent for use in Norwegian greenhouses in 2001. The risk of establishment outdoors was assessed too high and no permission was given. The first record in Norway was in 2006 when one adult was found on *Thuja* sp. imported from the Netherlands. In late 2007 and throughout 2008 adults were found indoors/outdoors at several locations in the Oslo-area. Establishment outdoors became evident. Observations in Aust-Agder, Vestfold and Trondheim revealed further spread/introduction to new areas. The bioclimatic potential of *H. axyridis* was assessed in 2007 by the aid of CLIMEX using national agrometeorological data, showing that suboptimal microclimates for the species can be found in the coastal areas of the south. In late 2008 a web-site was launched aiming to engage the public to submit observations on-line and has contributed to monitor development of the species across the country. Competition experiments with *H. axyridis* and *Coccinella septempunctata* in 2008/2009 showed that only *H. axyridis* eggs and larvae survived when the two species were mixed.

Key words: *Harmonia axyridis*, Norway, introduction, establishment, distribution, bioclimatic potential, CLIMEX

Introduction

The invasive harlequin ladybird *Harmonia axyridis* (Pallas, 1773) (Coleoptera: Coccinellidae) is known to be a voracious predator on aphids and has been extensively used as a biological control agent in both North America and Europe (Gordon, 1985; Ferran *et al.*, 1996; Katsoyannos *et al.*, 1997; EPPO, 2006). In later years, however, a number of negative impacts on the environment and biodiversity have been reported. The species was assessed as a potential bio-control agent for use in Norwegian greenhouses in 2001 (Statens landbrukstilsyn, 2001). It was concluded that *H. axyridis* might survive and establish outdoors and thereby pose a risk to the environment. The assessment was therefore negative with respect to import and commercial use in Norway. Nevertheless, the species found its way to Norway through other means and is now established in the country (Staverløkk *et al.*, 2007; Staverløkk & Sæthre, 2008).

H. axyridis has a wide diet and although it feeds mostly on aphids in all stages, it can successfully complete its lifecycle with the supply of scale insects (Coccidae), adelgids (Adelgidae), psyllids (Psylloidae), spider mites (Tedders & Schaefer, 1994; Hodek, 1996; Lucas et al., 1997; Koch, 2003). This species is able to reduce aphid numbers below economically damaging levels within many crop systems and thereby reducing the dependency of chemical pesticides (Roy *et al.*, 2006). Koch *et al.* (2006) lists arboreal hosts (trees and shrubs) and associated preys utilized by *H. axyridis*. More than 30 different species of aphids are listed and also a few species of Psyllidae, of which several are present in Norway.

During the last decade, considerably interest in all aspects of the biology of *H. axyridis* has been raised due to its rapid spread and establishment in Europe and North America as an alien and invasive species. The species is native to China, Japan, Korea, Mongolia and Siberia (Brown *et al.,* 2008). Numerous studies, both from the field and in laboratory have been published; for recent reviews, see Staverløkk *et al.* (2007) and Roy & Wajnberg (2008).

Material and methods

Stowaways in imported plant

In Norway, the import of horticultural plants for further cultivation in plant nurseries and particularly plants imported and sold directly to consumers for outdoor use in private gardens and urban landscapes has doubled between 1997 and 2006 (NGF, 2006). Therefore, a master thesis was carried out in 2006 with the aim to get an overview of the stowaways, the trade pattern and the control mechanisms involved in this business (Staverløkk, 2006).

Samples were taken from consignments of newly imported horticultural plants using three different sampling methods: 1. Shaking method (on the spot), 2. Visual detection (on the spot), and 3. Collecting soil and other organic material that had fallen off the consignments during transport (further investigations in the laboratory).

Assessing the Bioclimatic Potential of H. axyridis in Norway by the Aid of CLIMEX

As a response to the first records in Norway in 2006 and 2007 (see results below), the Match Climate function in CLIMEX was used to match the climate of four locations of origin for *H. axyridis* versus Norway and Europe (Staverløkk & Sæthre, 2007).

Two sets of climate response parameters for *H. axyridis* were developed, based on the requirements of a French and an American (Oregon, USA) population, respectively. Predicted world distribution maps were produced by the CLIMEX model (Compare locations, Sutherst *et al*., 2004) (Staverløkk & Sæthre, 2007).

In addition, the two climate response parameters developed where run in the CLIMEX model using Norwegian agrometeorological data, which are more representative for agroecological zones, private gardens and urban areas than the data stored in CLIMEX (Staverløkk and Sæthre, 2007). The parameters where run in CLIMEX both under the current climate scenario and under a “greenhouse climate scenario” (= 1°C increase in temperature).

Website on H. axyridis

In November 2008 Bioforsk Plant Health and Plant Protection Division in collaboration with the Directorate for Nature Management launched a website about *H. axyridis*: http://www.bioforsk.no/marihoner. The website provides information and pictorial guides of *H. axyridis* and some indigenous species in Norway. The general public is encouraged to submit their own observations of the new species by submitting an electronic form and a picture (or physical sample through ordinary mail). New records are verified by staff at Bioforsk and the distribution map updated accordingly (Figure 1 and 2).

Preliminary Competition Experiments

Cage experiments with the two species *H. axyridis* and *Coccinella septempunctata* together (competition cages) and separated (control cages) were conducted in the laboratory in 2008 and 2009. Experiments were conducted at 23°C, 70% RH, and the predators were fed with the aphid *Myzus persicae* on sweet pepper plants. In each cage eight unmated females and eight unmated males were inserted. The experiments went on until two generations had completed in each cage. The number of adults from each generation and each species was recorded. During the experiments other observations such as time of egg laying, egg hatch, larva and pupal stages in each cage were also recorded (Sæthre, unpublished data).

Results and discussion

Introduction, establishment and dispersal in Norway (Figure 1 & 2)

April 2006: The first record of *H. axyridis* in Norway was one adult female on *Thuja* sp. imported from the Netherlands. The specimen was found during sampling of consignments with horticultural plants newly imported from different countries in Europe (Staverløkk, 2006).

November/December 2007: Several live adults were found both outdoors and indoors at two locations downtown Oslo (Staverløkk & Sæthre, 2008).

January-December 2008: Several specimens recorded at 9 different locations both indoors and outdoors in Oslo. Two additional incidences of *H. axyridis* on imported *Thuja* sp. from the Netherlands were reported.

March 2008: Between 2000 and 3000 adult beetles were found in a cargo of plank imported to Åndalsnes (Møre og Romsdal County), from Pennsylvania, USA. Some specimens were also recorded at Snåsa (Nord-Trøndelag County), as some of the imported plank was transported on to this location. According to the importing company, actions were taken to eradicate the beetles, however, no further follow up has been taken to confirm whether the eradication procedure was successful or not.

July 2008: About 30 larvae and pupae were found in an Oslo park. This was the first important record on outdoor vegetation in Norway, suggesting that *H. axyridis* was able to survive the winter and had established a population in the area.

August 2008: Two larvae were collected from Tvedestrand, Aust-Agder, which was the first outdoor record outside Oslo. One additional location was discovered in Oslo when more than 30 larvae/pupae/adults were recorded in a garden area (Rodeløkka kolonihage).

September 2008: Several larvae and pupae were found on the vegetation at the lake Østensjøvannet in Oslo.

November 2008: One adult was found outdoors in a crack of a house (overwintering) at Våle, Vestfold.

2009: One adult was recorded in a private garden in Trondheim (about 600 km north of Oslo). This is the most northern record in Europe.

September 2009: Several larva and pupa where found on *Cornus* sp. infested with aphids in a park in Oslo (Torshovparken).

A detailed list of all Norwegian records until 10 November 2008 is given by Staverløkk & Sæthre (2008). However, the records from Tvedestrand, Våle (2008) and Trondheim (2009) are clearly indicating a further spread of the species in Norway than previously anticipated (Figure 1). The record from Trondheim leads to the conclusion that imported plants is likely to be the most important factor for introduction of the species to new areas. Natural geographic barriers and long distances within Norway limit the species possibilities for rapid natural dispersal.

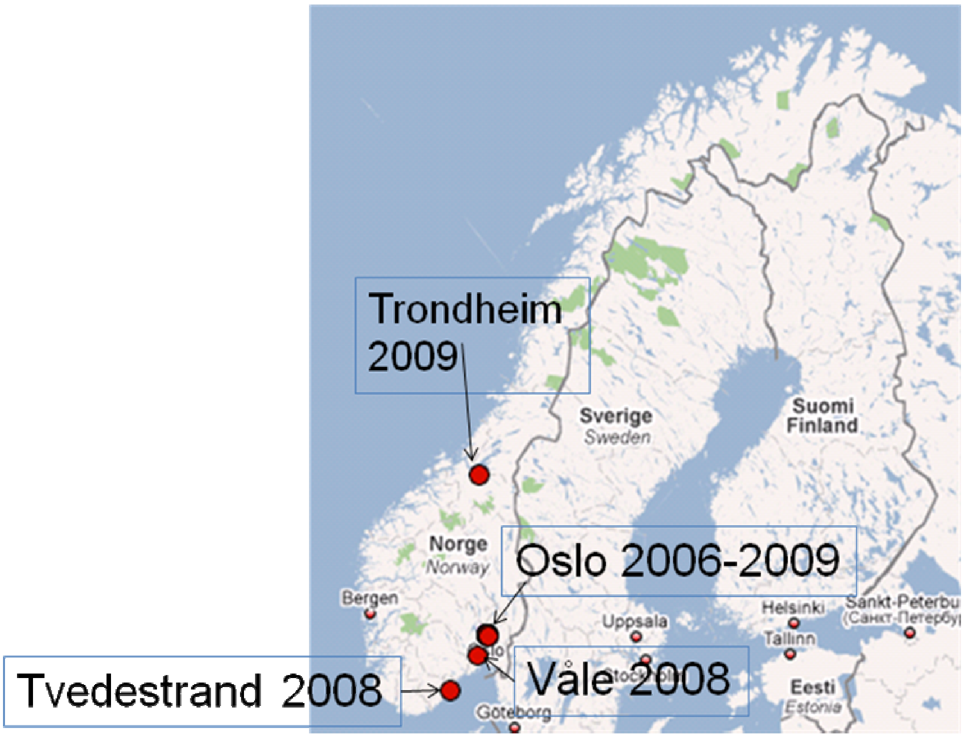

Figure 1. Distribution map of *Harmonia axyridis* in Norway per August 2009. (http://www.bioforsk.no/marihoner 2009)

Many of the plants imported for outdoor use have been grown in the field for one or several years in other parts of Europe (or elsewhere). These plants therefore become part of the site-specific-ecosystem where they are produced and serve as host plants, refugee sites and so on for organisms native to or present at that specific location. Plants grown directly in the field are in addition imported with a lump of the soil around their roots originating from the production site. This practice allows numerous organisms to enter new areas very easily and *H. axyridis* is only one of the species introduced in this way (Staverløkk & Sæthre 2007).

Assessing the Bioclimatic Potential of H. axyridis in Norway by the Aid of CLIMEX

Based on the Match Climates analysis it was concluded that the climatic conditions in several parts of Norway are within the range of the bioclimatic requirements for *H. axyridis* originating from Chita (Russia), and partly also from Vladivostock (eastern Russia) (Staverløkk & Sæthre, 2007).

The results obtained using the CLIMEX model for the two populations from France and Oregon, gave two quite different distribution maps, indicating that *H. axyridis* from populations with similar requirements as the Oregon population can establish on all continents and in a number of countries having a tropical or sub-tropical climate. The French population, however, showed a very limited distribution. According to the predictions none of the two populations were able to establish in Norway (Staverløkk & Sæthre, 2007).

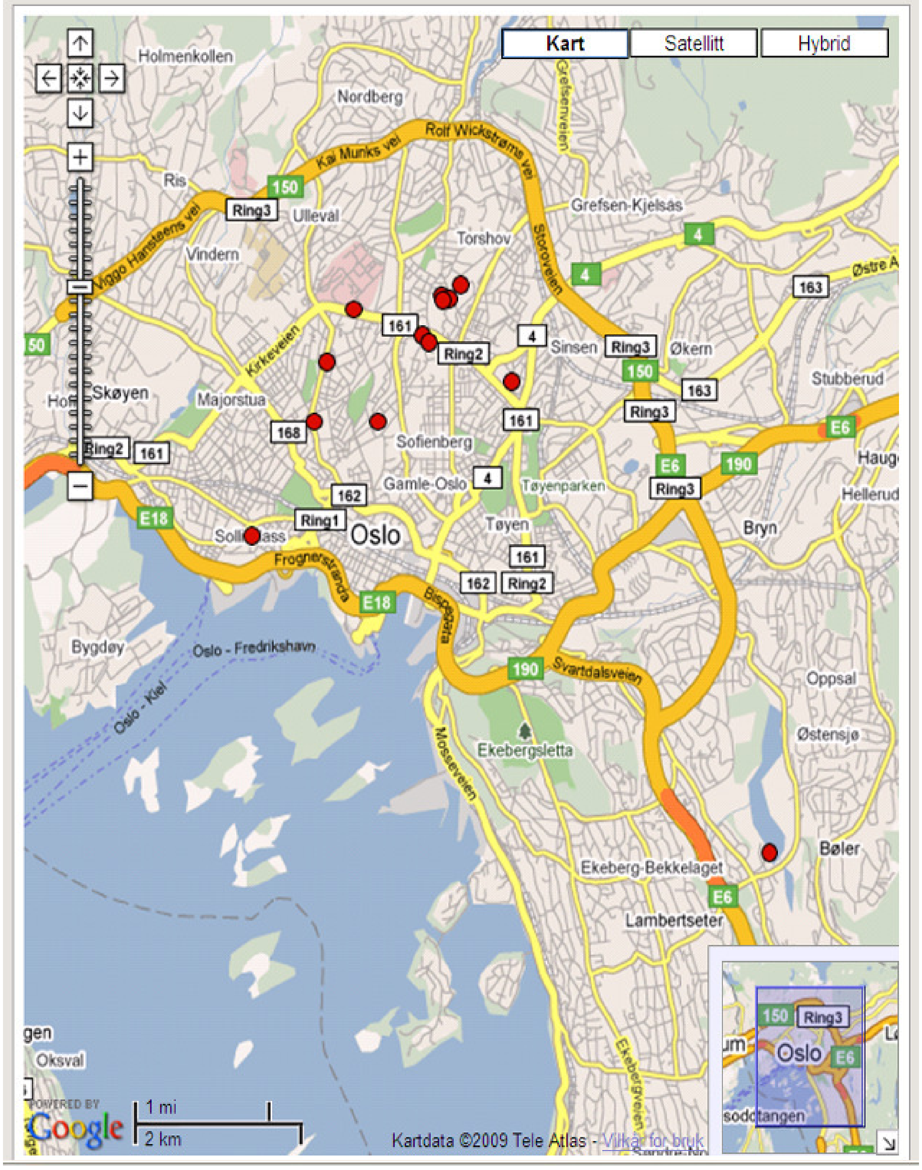

Figure 2. Distribution map of *Harmonia axyridis* in the Oslo-area per August 2009. (http://www.bioforsk.no/marihoner 2009)

When the same two climate response parameters developed where run in the CLIMEX model using Norwegian agrometeorological data, the results revealed that suboptimal microclimates for the species most certainly can be found at protected locations particularly in the coastal areas of the south-west of Norway under the current climate scenario (Figure 3). An increase in temperature of one degree Celsius (greenhouse climate scenario) lowered the cold stress in eastern Norway, but not to a level that could be ignored by the model (according to the requirements set for the species) (Figure 4).

As the climate response parameters for *H. axyridis* developed by Staverløkk & Sæthre (2007) excluded the areas in south eastern Norway where *H. axyridis* has later established, the parameters will be adjusted as new knowledge about the species behavior and distribution in Norway is obtained. Planned future studies on biology, ecology, cold tolerance and winter survival will contribute to a better prediction of dispersal and potential new areas for establishments of the species.

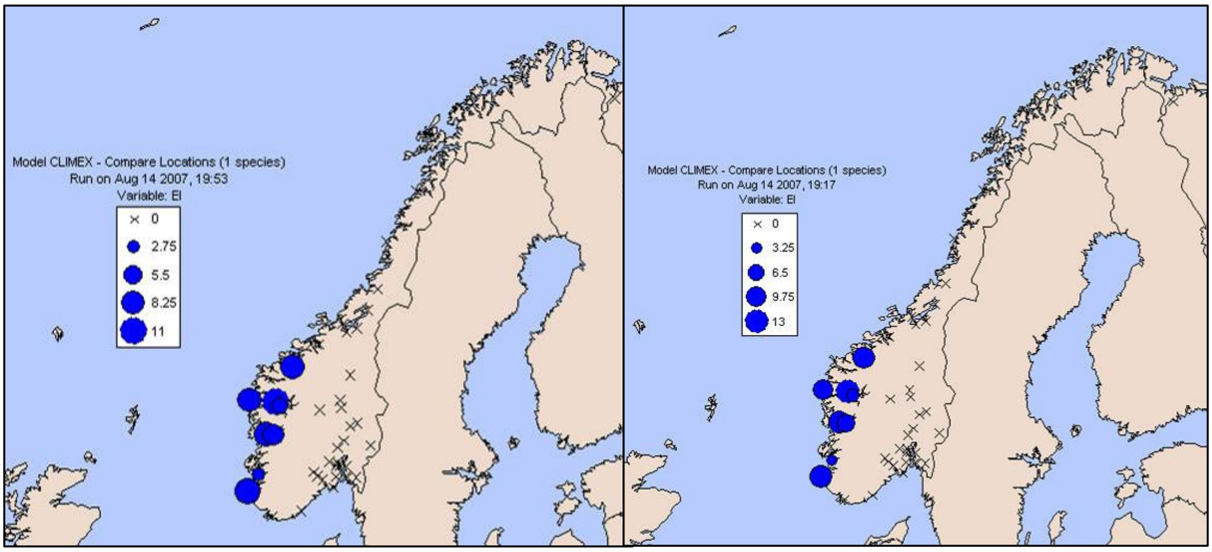

Figure 3. Ecoclimatic indices (EIs) for two populations of *Harmonia axyridis* in Norway under the current climate. Climatic response parameters based on the requirements of a population of *H. axyridis* from France (left map) and a population of *H. axyridis* from Oregon (USA) (right map). Model predictions made by CLIMEX.

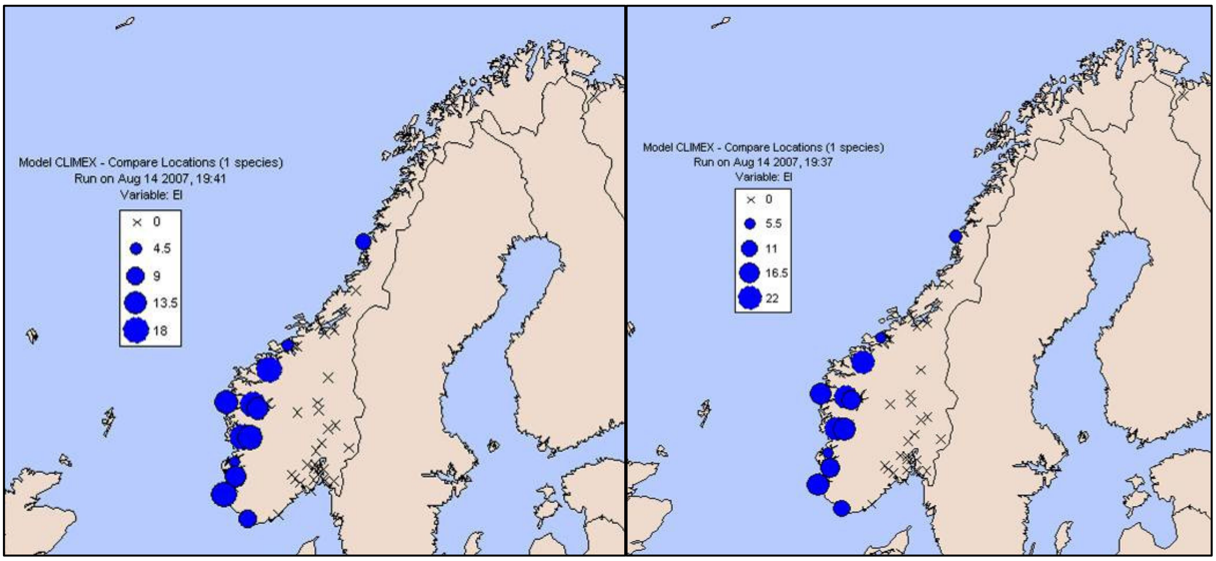

Figure 4. Ecoclimatic indices (EIs) for two populations of *Harmonia axyridis* in Norway under the greenhouse climate scenario (+1ºC). Climatic response parameters based on the requirements of a population of *H. axyridis* from France (left map) and a population of *H. axyridis* from Oregon (USA) (right map). Model predictions made by CLIMEX.

Preliminary Competition Experiments

Only *H. axyridis* eggs and larvae survived in cages where the two species were mixed (in one case one single larva of *C. septempunctata* was recorded to survive). In all other cases the eggs/young larvae of *C. septempunctata* were eaten, indicating a faster development time in *H. axyridis* and that these larvae are vicious predators on the eggs of *C. septempunctata* before they hatch (Sæthre, unpublished data). In the control cages *C. septempunctata* reproduced normally, but clearly had a slower development than *H. axyridis*. The results support what has been reported from other countries (Snyder *et al*., 2004; Pell *et al.,* 2008; Sato & Dixon, 2004). The mechanisms leading to the results are not studied and fully understood and should be looked into. The experiments will be repeated at different temperatures to see if perhaps lower temperatures can be an advantage for *C. septempunctata*.

Conclusion

H. axyridis is a species showing extreme ecological plasticity and has developed sub-populations with rather different climatic requirements than what should be expected when assessing the climate at its area of origin. The primary concern of *H. axyridis* in Norway is its impact as an alien invader on non-target arthropods (Koch & Galvan, 2008). Some negative impacts of *H. axyridis* as a household invader and as a pest in fruit production have been seen in other countries, but in this respect regarded as secondary problems only.

Acknowledgements

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Harmonia axyridis as a model for predator adaptation to chemically defended prey

John J. Sloggett[1], Kenneth F. Haynes[2], John J. Obrycki[2] & Andrew J. Davis[3]
[1]*Tussen Beide Markten 45, 9712 CC Groningen, The Netherlands;* [2]*University of Kentucky, Department of Entomology, S-225 Agricultural Science Center North, Lexington, KY 40546-0091, USA;* [3]*Max Planck Institute for Chemical Ecology, Beutenberg Campus, Hans-Knöll-Straße 8, 07745 Jena, Germany*
Corresponding author: johnsloggett@yahoo.co.uk

Abstract: *Harmonia axyridis* is a stronger intraguild predator of other ladybirds than are many other ladybird species. A correlate of this is that *H. axyridis* is better at resisting the toxic effects of the alkaloids of allospecific ladybird prey. This makes *H. axyridis* an ideal species with which to investigate the adaptations of predators to feeding on potentially toxic chemically defended prey. In this paper we discuss recent studies that, for the first time, have thrown light on the nature of and mechanisms involved in *H. axyridis* alkaloid resistance. We focus on the finding that *H. axyridis* is relatively poorly adapted to prey containing novel alkaloids in areas where it is exotic and on the fate of suitable and unsuitable prey alkaloids after ingestion by the intraguild predator.

Key words: alkaloids, Coccinellidae, coevolution, detoxification, *Harmonia axyridis*, intraguild predation, physiological adaptation

Introduction

Many organisms are protected against potential consumers by chemical compounds that render them unpalatable or toxic. Natural enemies that consume these organisms must possess adaptations to counter or circumvent their defensive chemistry. These have been most thoroughly investigated in the context of plant-herbivore interactions, and we know a lot about the mechanisms by which herbivores tolerate plant allelochemicals and even use them to their advantage. In contrast, predator adaptations to chemically defended prey have been poorly studied. Most studies related to prey chemistry focus on predator decision making and the avoidance of such prey, rather than on how predators may adapt to exploit this resource.

Many predators do incorporate chemically defended prey in their diet, nonetheless. There is a substantial body of evidence that the ladybird *Harmonia axyridis* readily consumes other aphidophagous ladybird species in the field (intraguild predation, IGP); this is typically predation by *H. axyridis* larvae of other species' eggs, larvae or pupae (e.g. Takahashi, 1989; Hironori & Katsuhiro, 1997; Cottrell & Yeargan, 1998; Toda & Sakuratani, 2006; Hautier *et al.*, 2008, 2010; Ware *et al.*, 2009). Ladybirds utilise taxonomically variable autogenous alkaloid defences (Daloze *et al.*, 1995) and the different ladybird species in a community generally possess different alkaloids (e.g. Sloggett *et al.*, 2009a); consequently when *H. axyridis* eats another species it is generally exposed to alkaloids it does not possess itself. For ladybirds that are weaker intraguild predators, allospecific ladybird alkaloids are often toxic when they are consumed (Hemptinne *et al.*, 2000; Cottrell, 2004). In contrast, *H. axyridis* appears to suffer fewer ill-effects when feeding on other ladybird species (e.g.

Yasuda & Ohnuma, 1999; Cottrell, 2004; Sato *et al.*, 2008). In other words, because it is a habitual intraguild predator, *H. axyridis* has evolved resistance to the prey alkaloids it ingests.

Because of its well-established alkaloid resistance, *H. axyridis* makes an ideal organism with which to study predator adaptation to chemically defended prey. We can address questions about the specificity of its adaptations and the mechanisms it uses to counter alkaloid toxicity. A particularly valuable feature of *H. axyridis* for these studies is its exotic status in many areas: because the species has colonised new regions, we can examine how it responds to novel prey with alkaloids to which it has not previously been exposed.

Studies of *H. axyridis*' alkaloid resistance outside its native range

IGP studies have tended to suggest that *H. axyridis* is good at dealing with ladybird prey alkaloids *per se* and have not considered shared evolutionary history or have concluded that there are few differences between prey inside and outside of *H. axyridis*' native range (e.g. Cottrell, 2004; Sato & Dixon, 2004; Rieder *et al.*, 2008; Ware *et al.*, 2008). Most such studies were more general studies of IGP, not specifically related to prey chemical defence and in some cases the prey alkaloids were unknown. In some studies there was a focus on reciprocal testing (i.e. *H. axyridis* preying on other species and these species preying on *H. axyridis*): in these cases, it is clear that *H. axyridis* performs better on the other species than they do on *H. axyridis*. However, this is a most likely because *H. axyridis* has itself evolved strong chemical defences against IGP, particularly as it is abundant when aphid prey are declining or scarce (Sato & Dixon, 2004). Furthermore, even highly alkaloid-specific resistance is effective if prey in newly-colonised areas share alkaloids with prey in *H. axyridis*' native range.

In order to better test how prey with novel chemical defences affect the performance of *H. axyridis*, Sloggett *et al.* (2009b) fed eggs of five different ladybird species of Kentucky field crops to (exotic) first instar North American *H. axyridis* larvae. The five prey species all possessed known chemical defences (Fig. 1) and comprised *H. axyridis* itself, one other North American exotic that occurs in *H. axyridis*' native range (*Coccinella septempunctata*: "coevolved prey" of Fig. 1) and three North American native species (*Hippodamia convergens*, *Coleomegilla maculata* and *Cycloneda munda*: "non-coevolved prey" of Fig. 1). Pea aphids, *Acyrthosiphon pisum*, were used as a control diet. Larvae of *H. axyridis*, which showed little initial discrimination between the different prey, were monitored until the second instar and mortality and developmental time were used as prey suitability indicators. Considered overall, the performance of *H. axyridis* larvae was best on a diet of conspecific eggs, followed by those of *H. convergens*; *C. septempunctata* eggs and aphids were of similar slightly lower suitability, and *C. munda* and *C. maculata* eggs were the least satisfactory, the former causing lengthened development and the latter also causing high mortality. The lengthened development on *C. munda* eggs was because they were highly unpalatable to the larvae, which were reluctant to eat them. The eggs of *C. maculata* were toxic, causing larval paralysis for long periods after consumption, and, on an exclusive *C. maculata* egg diet, death in about half of the larvae. Larval paralysis after *C. maculata* egg consumption meant that larvae provided with a single egg were unable to forage for aphids for long periods after eating it, unlike those fed other types of prey.

Thus of the three American native prey only one, *H. convergens*, was truly suitable, while the other two were either unpalatable (*C. munda*) or toxic (*C. maculata*). Prey suitability appears to be related to alkaloid content and evolutionary history. Performance on *C. septempunctata*, which occurs in *H. axyridis*' native range and to which *H. axyridis* expected to be adapted was moderately good, as in earlier studies (Yasuda & Ohnuma, 1999;

Sato & Dixon, 2004; Rieder *et al.*, 2008). The one suitable American native species, *H. convergens*, contains an alkaloid possessed by *H. axyridis* itself, harmonine, and the structurally similar n-octylamine; neither alkaloid is likely to be very toxic to *H. axyridis*. The other alkaloids of *H. convergens* are hippodamine and convergine, which are stereoisomers of precoccinelline and coccinelline from *C. septempunctata*. Hippodamine and convergine are widespread in the aphidophagous subfamily Coccinellinae one or both having been described from five genera, including the cosmopolitan *Hippodamia* genus (Sloggett *et al.*, 2010 and references therein); it thus seems likely that *H. axyridis* encounters prey with these alkaloids in its native range. A further possibility is that it uses the same mechanism to counter their toxicity as for precoccinelline and coccinelline.

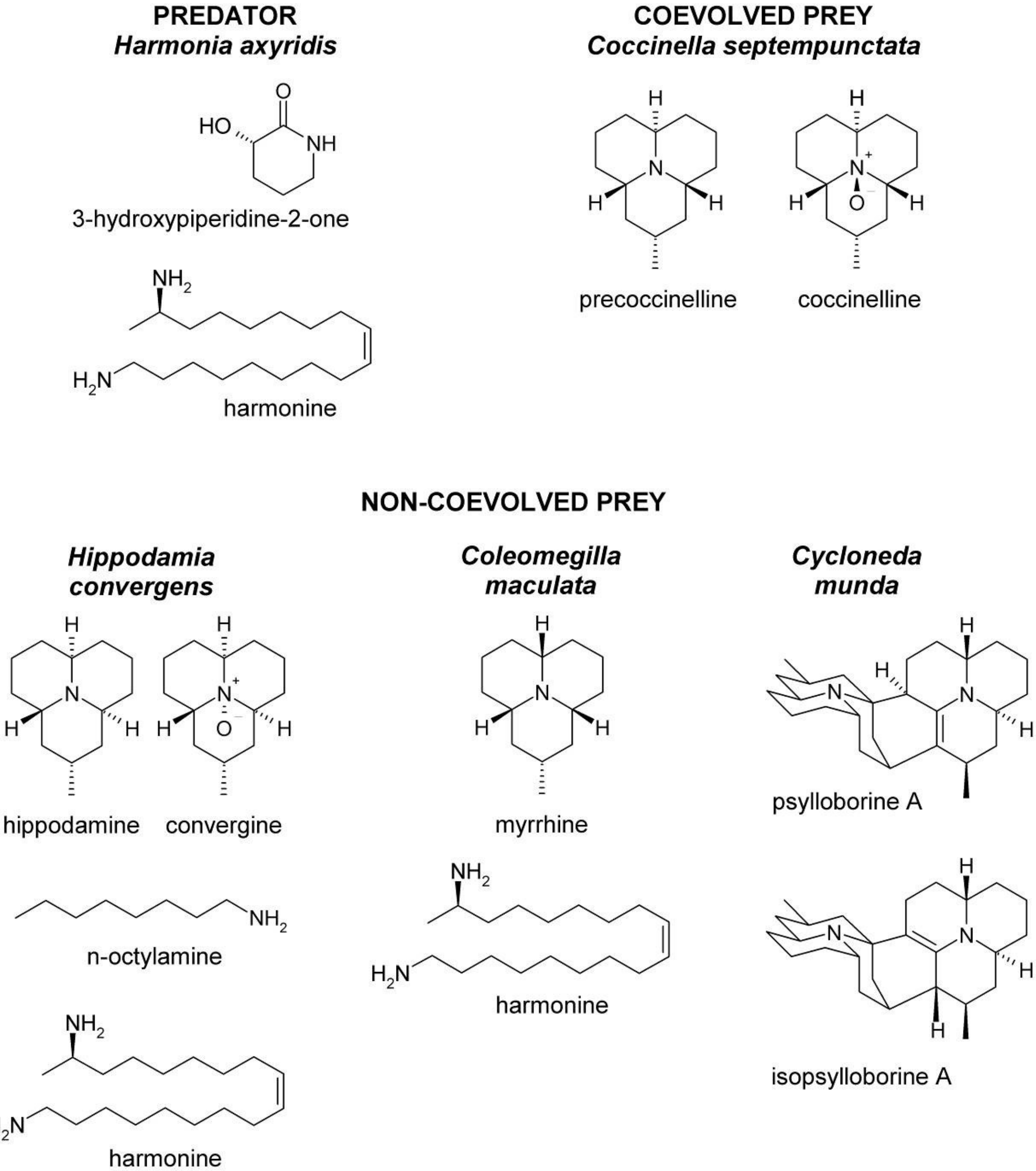

Figure 1. Ladybird species used in the feeding experiments of Sloggett *et al.* (2009b) and their alkaloids. Alkaloid identifications from Sloggett *et al.* (2009a) and references therein.

The two remaining species, *C. munda* and *C. maculata* are from genera restricted to the American continent, and in both cases their alkaloids are taxonomically uncommon. The larger alkaloid molecules of *C. munda* are characteristic of mycophagous, but not aphidophagous genera and, while *C. maculata* possibly contains harmonine, myrrhine is elsewhere limited to the Palaearctic pine specialist *Myrrha octodecimguttata*. Thus, in neither case is *H. axyridis* likely to regularly encounter species containing these alkaloids in its native range. It thus seems probable that its poor performance on these species is because in its native range it did not face the selective pressure to adapt to the alkaloids they possess.

From a physiological viewpoint, this study tells us something about the specificity of *H. axyridis*' adaptations to prey chemistry. Given this species sheer dietary breadth, its adaptations are surprisingly specialised. This is best shown by the cases of precoccinelline, hippodamine and myrrhine; these three alkaloids are all stereoisomers of each other, arguably one of the smallest structural differences possible. Nonetheless while *C. septempunctata*, containing precoccinelline, and *H. convergens*, with hippodamine, are suitable prey, the myrrhine-bearing *C. maculata* is toxic. The study is also informative from an ecological viewpoint. In the context of invasive species, the mismatch between the adaptations of consumers and the chemical defences of the consumed is more typically considered as an advantage to invasive organisms, sometimes reducing pressure from potential natural enemies in their new range. Cases are known of chemically defended invasive prey which are toxic to native predators, when they consume them (e.g. Phillips *et al.*, 2003; Suttle & Hoddle, 2006). This study shows that this mismatch does not always work in the invader's favour: when the invader is the consumer, its food may present significant novel chemical challenges.

The physiological basis of *H. axyridis*' alkaloid resistance

The previous study gives us some insight into the nature of *H. axyridis*' physiological adaptations to prey alkaloids, but does not tell us much about what these physiological adaptations might be. Such studies are still in their infancy, but we are now beginning to gain some idea of what these adaptations are. The same chemical techniques which are used to identify and measure chemical defences in organisms can also be used to examine their fate in the consumers of that organism (e.g. Becerro *et al.*, 2006; McGovern *et al.*, 2006). Using gas chromatography-mass spectrometry (GC-MS) it has proved possible to visualise and measure many prey ladybird alkaloids within the body of *H. axyridis* after consumption (Fig. 2). Interest in this method was initially more focused on its use for determining IGP by *H. axyridis* in the field (Hautier *et al.*, 2008, 2010; Sloggett *et al.*, 2009a). However, it can also provide important information on the *H. axyridis*' physiological adaptations to prey alkaloids through ADME (absorption, distribution, metabolism and excretion) studies, that is studies of the occurrence and abundance of alkaloids in the predator body and its different parts after alkaloid-containing prey have been consumed.

Such a study has recently been carried out by two of us (Sloggett & Davis, 2010). Unlike the previous American study, this work used European *H. axyridis* as predators and, building on the findings of the earlier study, included a novel European prey, *Adalia bipunctata*. This species historically did not occur in *H. axyridis*' native range (although occurs there now: Sakuratani *et al.*, 2000; Toda & Sakuratani, 2006). Although a regular prey of *H. axyridis* where two species now co-occur (e.g. Sakuratani *et al.*, 2000; Toda & Sakuratani, 2006; Hautier *et al.*, 2008, 2010; Ware *et al.*, 2009), *A. bipunctata* is quite toxic to the intraguild predator (Sato & Dixon, 2004), presumably due to the lack of historical coexistence of the two species. A second, more suitable prey, *Propylea japonica*, was compared to *A. bipunctata* in the ADME study. *Propylea japonica*, an intraguild prey of *H. axyridis* in its native range, had previously been shown to be a suitable diet for *H. axyridis* in feeding experiments (Sato *et al.*, 2008); presumably, *H. axyridis* is physiologically adapted to *P. japonica*'s alkaloids.

The study aimed to deduce some of the physiological adaptations of *H. axyridis* to prey alakloids by comparing the ADME profile of isopropyleine, the major alkaloid of *P. japonica* with that of adaline, the major alkaloid of *A. bipunctata*. Fourth instar *H. axyridis* larvae were fed three eggs of one or other prey species and frozen for GC-MS analysis either immediately after they had eaten the eggs or 4, 8, 16 or 32 h after egg consumption. Haemolymph samples were collected from larvae over the time periods, and all frass produced by the larvae up to 32 h was also collected and analysed (Sloggett & Davis, 2010).

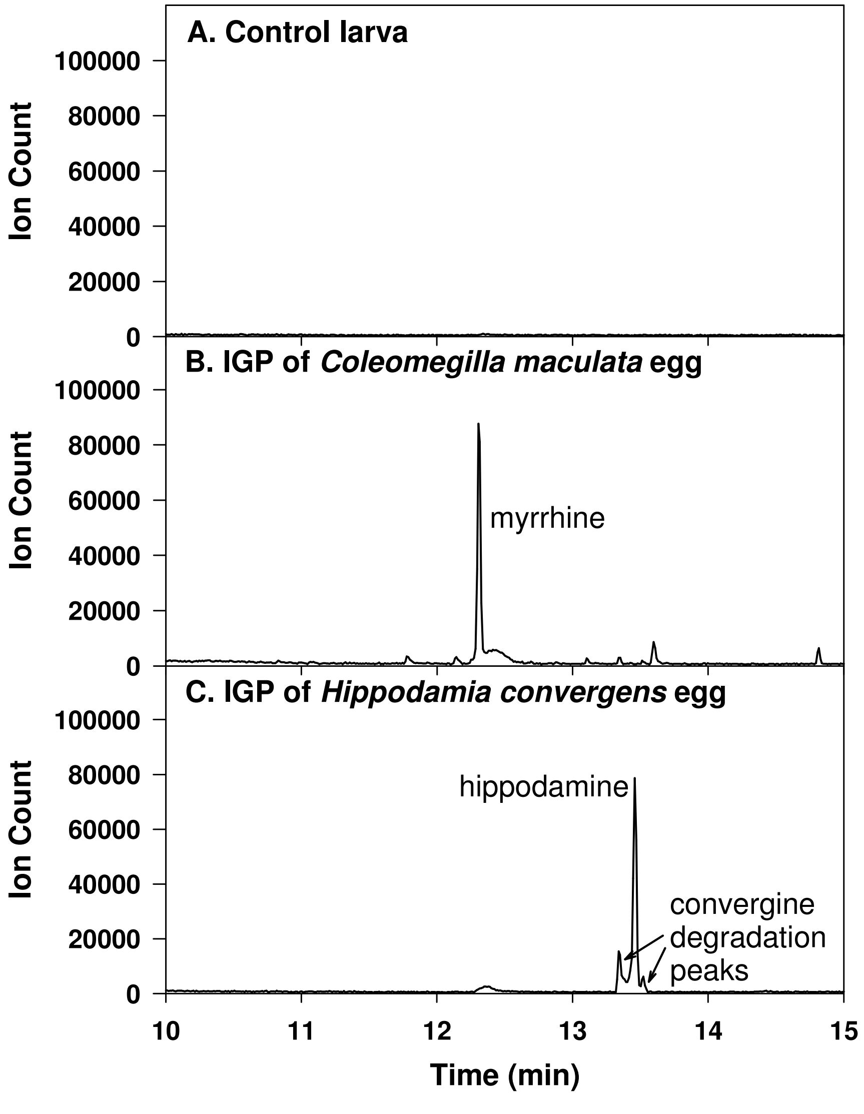

Figure 2. Gas chromatograms of extracts of whole third instar *H. axyridis* larvae showing the alkaloids of (North American) prey that they have eaten. (A) a *H. axyridis* larva that has not eaten any ladybird prey; (B) one that has eaten a *C. maculata* egg, showing a myrrhine peak; (C) one that has eaten a *H. convergens* egg, showing hippodamine and degraded convergine peaks. Note that *H. axyridis* alkaloids and n-octylamine from *H. convergens* do not appear in the GCs as they are too polar for this analysis. From Sloggett *et al.* (2006).

The isopropyleine of *P. japonica*, to which *H. axyridis* is adapted, declined in the bodies of the larvae more quickly than did the the novel alkaloid adaline of *A. bipunctata*. While <1% of the initial amount of isopropyleine consumed remained after 32h, >10% of adaline remained. Both alkaloids entered the predator's haemolymph; however, isopropyleine entered the haemolymph immediately after egg consumption and disappeared after 4h, while adaline did not appear in the haemolymph until 4 h after egg consumption and was detectable in some samples even 32h after egg consumption. The amount of alkaloid in frass was relatively low: although 32h is long enough for food to pass entirely through the gut, frass contained on average <10% of isopropyleine consumed and <20% of adaline consumed.

The results indicate that the alkaloids are primarily chemically altered within the predator body rather than being excreted unchanged. This occurs more rapidly in the case of isopropyleine, suggesting specialised metabolic pathways used by *H. axyridis* to alter this alkaloid to reduce its toxicity. By contrast adaline, to which *H. axyridis* is not adapted, is probably ultimately altered by non-specific general processes; it thus persists for longer, increasing its toxicity. Interestingly both alkaloids are transported into the haemolymph. This is particularly noteworthy for isopropyleine, indicating that the site of detoxification for this alkaloid is not the gut as is often the case in insect allelochemical metabolism, for example in many herbivores. Isopropyleine is transported into the haemolymph immediately after *P. japonica* eggs are consumed; possibly this transport into the haemolymph is an active process, while again for adaline it is a passive, non-specific process, taking longer. This study is the first to provide clear support for the existence of specialised metabolic pathways for the detoxification of prey alkaloids in *H. axyridis*. Such pathways only exist for the alkaloids of prey commonly consumed in its native range. In contrast no such pathways exist for novel alkaloids which are more toxic as they persist in the body for longer unchanged.

An earlier study by Hautier *et al.* (2008), using adaline as a marker for IGP of *A. bipunctata*, suggested that *H. axyridis* sequestered alkaloids from its ladybird prey for its own use. This conclusion was based exclusively on findings for adaline from single *A. bipunctata* neonate prey, which could persist in *H. axyridis* larvae for many days after the neonate was consumed. However, there was no corresponding data in the study for other prey species with different alkaloids. Sloggett *et al.* (2009a), using hippodamine from *H. convergens* also as a marker to detect *H. axyridis* IGP, concluded that prey alkaloids were not sequestered by the predator, as hippodamine from a single consumed egg persisted on average only 13.5 h. However, direct comparisons of the two studies are confounded by the differing experimental protocols and ways that data were reported, and probable differences in the concentrations of the two alkaloids in the prey (hippodamine being a more minor component than adaline). The ADME study is unequivocal that prey alkaloid is not sequestered by *H. axyridis*, at least in its original form (Sloggett & Davis, 2010). Sequestration is not the mere persistence of defensive chemicals from food in a consumer, but itself requires considerable evolutionary adaptation, to faciltate efficient allelochemical storage while minimizing toxic effects (e.g. Hartmann, 2004). If prey alkaloid sequestration occurred in *H. axyridis*, higher persistence would be expected for the alkaloids of historical prey, like *P. japonica*, to which *H. axyridis* has had time to adapt. This is the opposite of what is observed. The persistence of adaline coupled with its known toxicity is more suggestive of a nonadaptive inability to get rid of the alkaloid than an adaptive means of providing *H. axyridis* with additional chemical defence.

Conclusions

Harmonia axyridis is an ideal model for studying predator physiological adaptation to prey chemical defence. A huge body of work on *H. axyridis* ecology already exists and there are added opportunities provided by its alien status. Ladybird alkaloids are well studied and the chemical defences of many of its ladybird prey are thus already well known: consequently, physiological studies are not inhibited by poor knowledge of prey chemistry, in contrast to the situation for many aphid prey. There is already enough evidence to suggest that *H. axyridis* chemically alters prey alkaloids to render them less toxic and that such metabolic mechanisms are relatively alkaloid-specific. Future work is likely to focus on the biochemical pathways by which this is achieved, on other means of alkaloid resistance, such as target site insensitivity, and on the potential for *H. axyridis* to adapt to novel alkaloids of prey where it is invasive.

In the case of prey with novel, toxic alkaloids, both Cottrell (2004, 2005) for *C. maculata* and Ware *et al.* (2009) for *A. bipunctata* have emphasised that under certain conditions such as aphid scarcity, consumption of such prey can be beneficial for *H. axyridis*. The conclusions of their ecological studies are not at odds with the physiological studies described here, in spite of the apparent conflict. Although in relative terms prey ladybirds with novel alkaloids are of poor quality compared to prey with alkaloids to which *H. axyridis* is adapted, conditions such as aphid scarcity will make feeding on such prey less costly or beneficial. Further work is required to examine the interaction between the ecological and physiological aspects of *H. axyridis* IGP and how this may impact on the invader in the field.

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Colour pattern polymorphism and chemical defence in *Harmonia axyridis*

John J. Sloggett
Tussen Beide Markten 45, 9712 CC Groningen, The Netherlands
E-mail: johnsloggett@yahoo.co.uk

Abstract: Chemical defence has long been considered to play some sort of role in relation to colour pattern polymorphism in ladybirds. A recent idea is that intraspecific variation in colour or pattern is an indicator of the strength (i.e. concentration) of defensive chemicals in the individual. This has received support from a recent study showing that in non-melanic *Harmonia axyridis* the proportion of the elytra that is orange is positively correlated with the concentration of the alkaloid harmonine. In this paper I discuss palatability experiments with *H. axyridis* designed to test whether the finding can be extended across colour pattern morphs, specifically whether melanic *H. axyridis* are less well defended than non-melanics. Feeding experiments using spiders (*Araneus diadematus*) and earwigs (*Forficula auricularia*) gave no indication that melanics were less well defended than non-melanics. However, the spiders exhibited a generally high level of acceptance of ladybird prey, making the detection of intraspecific differences in prey palatability unlikely, while data from earwigs is currently of too small a scale to be unequivocal. Further palatability tests are required as well as additional analytical work covering the full range of *H. axyridis* morphs and defensive chemicals.

Key words: chemical defence, colour pattern, *Harmonia axyridis*, melanism, polymorphism, predation

Introduction

Colour pattern polymorphism in ladybirds has fascinated biologists for over a century (Komai, 1956; Majerus, 1994; Honěk, 1996). The realisation that conspicuous polymorphisms can be maintained by a balance of strong selective forces (Ford, 1964) has led to a plethora of studies on ladybirds aimed at identifying what these forces are. They appear to be diverse and are believed to include climatic factors, pollution, and non-random mating and sexual selection (Majerus, 1994; Honěk, 1996). A link between ladybird colour pattern polymorphism and chemical defence has long been considered. This is hardly surprising, as the bright warning colours of ladybirds advertise that they are distasteful or toxic due primarily to their alkaloid chemical defences. Brakefield (1985) proposed that different morphs of *Adalia bipunctata* and *Adalia decempunctata* might be members of separate Müllerian rings, mimicking different ladybird species. This idea was tested in a number of subsequent papers focusing on the palatability of different ladybird species and their relative investment in chemical defence (Marples *et al*., 1989; Holloway *et al*., 1991; de Jong *et al*., 1991; Marples, 1993a).

The work of Brakefield and colleagues focused largely on interspecific differences in ladybird chemical defence and their relationship to polymorphism. A more recent idea is that intraspecific differences in colour pattern might be indicators of chemical defensive strength, most notably the amount (concentration) of alkaloid in different individuals (Bezzarides *et al*., 2007). Early work on ladybird chemical defence showed that the alkaloid of different morphs of *A. bipunctata* was structurally the same (Tursch *et al*., 1973), but although it was known that individual ladybirds varied in the quantity of alkaloid they possessed (e.g. Holloway *et al*, 1991; de Jong et *al*., 1991), a link to colour pattern had not previously been considered. In the first and currently only published study of its kind, Bezzarides *et al.* (2007) examined alkaloid concentration in different individuals of North American *Harmonia axyridis* in relation to their colour and pattern. The study was limited to non-melanic ladybirds due to the rarity of melanic morphs in North America, and only one of the two alkaloids of *H. axyridis*, harmonine, was quantified (the other alkaloid being 3-hydroxypiperidine-2-one: Alam *et al*., 2002). Five elytral colour-related parameters were measured for males and females: orange hue prior to carotenoid extraction, carotenoid concentration, post carotenoid-extraction orange hue (from pterin pigments), black spot brightness and area of orange and black colouration (equivalent to spot number and size). There was no significant correlation between alkaloid content and pre- or post-extraction orange hue or carotenoid concentration, and spot brightness was negatively correlated with alkaloid concentration in females only. However total alkaloid concentration was correlated strongly with the percentage area of orange in the elytra of females and a similar, though not significant, trend was also found in males.

Bezzarides *et al.* (2007) hypothesise that a heightened proportion of the elytra covered in orange is an indicator of greater unpalatability, and discuss the implications of their study for other morphs of the ladybird, which clearly differ more widely in the proportion of orange displayed. They argue that lower defence levels in melanics could be offset by other advantages, such as increased activity rates through a thermal advantage. However, as they point out, further such studies are required of other *H. axyridis* morphs to examine whether the findings from non-melanics can be extended to them.

In this paper I discuss the first tests of whether melanic *H. axyridis* are indeed less well defended against natural enemies than non-melanics. In these experiments rather than an analytical approach, the palatability of melanics and non-melanics was tested. In the final discussion, I consider how palatability, chemical defence and colour pattern might be inter-related in *H. axyridis*.

Experimental work with spiders, *Araneus diadematus* (Araneae: Araneidae)

Initial comparisons of the defensive capabilities of melanic and non-melanic *H. axyridis* used the garden cross spider, *Araneus diadematus*, as a predator (Sloggett, 2010). This spider is a common native European web-spinning species, which had previously been observed to eat ladybirds in the field (Majerus, 1994). Spider predation of ladybirds has been relatively poorly studied and the study was more generally aimed at elucidating the nature of the relationship between *A. diadematus* and putative ladybird prey, with both a laboratory and field component. In the laboratory, Dutch *A. diadematus* were provided with prey with no chemical defences (a cricket, *Acheta domesticus*), an adult of the native European ladybird *Coccinella septempunctata* or a melanic or non-melanic adult of the exotic *H. axyridis* (Fig. 1). Surprisingly, the spiders showed relatively little aversion to ladybird defensive chemistry. Ladybirds of both species were captured and eaten by the spiders (e.g., see Fig. 1), and tests of heterogeneity across all treatments were not significant either for capture or consumption of prey. Of the three ladybird prey, non-melanic *H. axyridis* were most often eaten (15 captured out of a total of 39 matched replicates, with 13 completely eaten; Fig. 1), followed by melanic *H. axyridis* (12 captured and 11 completely eaten). No spiders exhibited any obvious toxic effect after eating any of the ladybirds. In the field, both *H. axyridis* forms were found in *A. diadematus* webs, although at too low a frequency for any comparative analysis. Ten spiders provided with non-melanic *H. axyridis* in the field were all later observed eating the ladybirds; however, no melanics were used in this experiment (Sloggett, in press).

The two forms of *H. axyridis* do not appear to differ in their chemical defensive capabilities against *A. diadematus*. In fact, marginally (and non-significantly) more non-melanics than melanics were captured and eaten in the laboratory experiment, the opposite trend to that which would be expected from the arguments of Bezzarides *et al.* (2007). However, it was also apparent that, more generally, ladybird chemical defences have little deterrent or toxic effect on *A. diadematus*: the spider is therefore unlikely to be sensitive to intraspecific differences in ladybird alkaloid concentration. From the perspective of *H. axyridis*, perhaps the most interesting aspect of this study is that in spite of a lack of coevolutionary history between the spider and the ladybird, the spider is unaffected by *H. axyridis* chemical defences, which include the novel alkaloid, 3-hydroxypiperidine-2-one, not thought to occur in the native European ladybird fauna. This forms an interesting counterpoint to other studies, where non-coevolved predators perform poorly on prey with novel chemical defences (see Sloggett *et al.*, 2010; Suttle & Hoddle, 2006 for a spider example).

Figure 1. The experimental set-up used in laboratory feeding tests with the spider *Araneus diadematus* (Sloggett, in press). Spiders were confined in 250ml plastic cups with live prey. The dead remains of a non-melanic *Harmonia axyridis* adult (of which all the soft parts have been eaten) are indicated with an arrow.

Work with earwigs (Dermaptera)

It is clear from work with *A. diadematus* that not all predators are suitable for comparison of melanic and non-melanic *H. axyridis* acceptability and toxicity. Some, probably including the spider, are unaffected by the ladybird's chemical defences irrespective of strength, and are thus unsuitable for intraspecific comparative work. At the opposite end of the spectrum, other potential natural enemies may find the defensive chemistry so unacceptable or toxic that they refuse to attack or consume the ladybird, or are very deleteriously affected if they do. Such consumers are also unlikely to be sensitive to differences in natural concentration, although in this case they can often be tested by mixing prey material and other food substances, diluting defensive substances below natural concentrations by a fixed amount.

The ideal consumer for examining intraspecific differences in alkaloid concentration lies between these two extremes. That is, it suffers repellent or toxic effects, but these are not so strong that it invariably refuses to eat the prey, especially when hungry. Earwigs, which are omnivorous, fall into this category. When satiated, common earwigs, *Forficula auricularia*, refuse to eat *H. axyridis* cadavers; however, when starved they will consume the cadavers in spite of the chemical defences (Fig. 2). They thus make a suitable "model" consumer with which to test hypotheses about morph-related intraspecific differences in *H. axyridis* chemical defence. It should be noted that earwigs are unlikely to consume adult ladybirds under natural conditions, although they are believed consume ladybird eggs (Majerus, 1994).

Work comparing the acceptability of melanic and non-melanic *H. axyridis* based-food for earwigs is described for the first time here. It has not progressed past preliminary testing, and consequently results must be considered tentative at the present time.

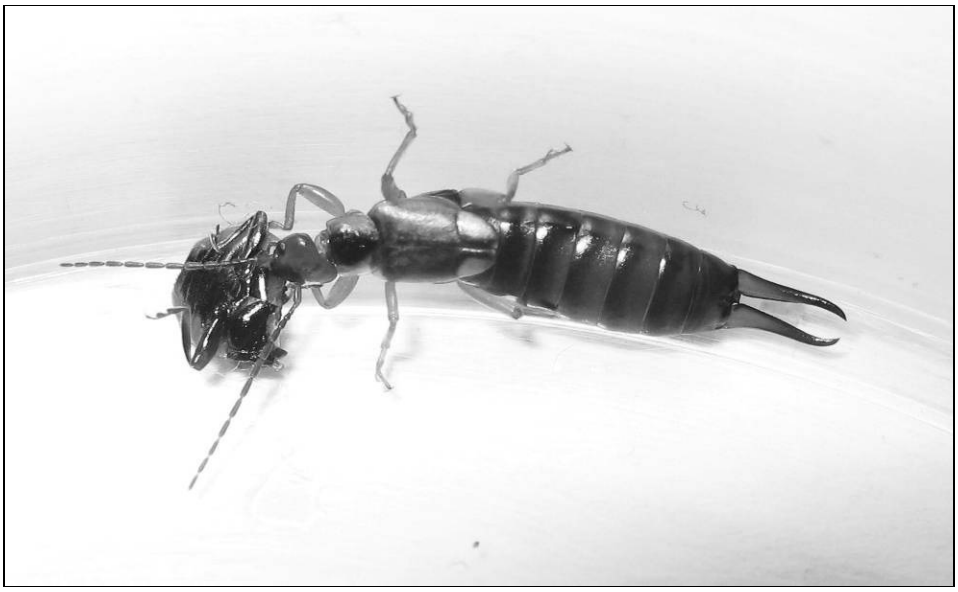

Figure 2: A starved common earwig, *Forficula auricularia*, feeding on the soft tissue from an excised *Harmonia axyridis* abdomen in the laboratory.

Material and methods

A basic food mixture was prepared in a food blender from approximately 400g ground beef, 45g bread and 5g honey. Into 150g of this mixture, either 15 melanic or 15 non-melanic female *H. axyridis* that had been killed and stored frozen (at -15°C) were blended, giving a concentration of approximately 0.5% *H. axyridis* by mass. After preparation, the mixtures were stored frozen at -15°C. Earwigs were collected from sites around the city of Groningen in the Netherlands (as were the ladybirds). Prior to experimentation they were maintained on a diet of old fruit and vegetables, and the basic food mixture without any ladybird content.

For experiments earwigs were isolated without access to either food or water for 4-5h. Preliminary tests indicated that after this treatment a 0.5% *H. axyridis* mixture was slightly less attractive to the earwigs as food than an uncontaminated control mixture. For the experiment, individual earwigs were placed in small arenas made from the bottom of plastic egg boxes and covered with a clear plastic lid. A small lump of either the melanic or non-melanic mixture covered most of the bottom of the arena, such that the earwig came into immediate contact with it (Fig. 3). The earwig was observed for 10 minutes and the period of time it spent feeding was recorded. Experiments were conducted as paired trials, with two earwigs tested with one or other of the two mixtures, one after the other. The length of time feeding on the two mixtures was compared using a two-tailed paired *t*-test in SPSS 8.0.

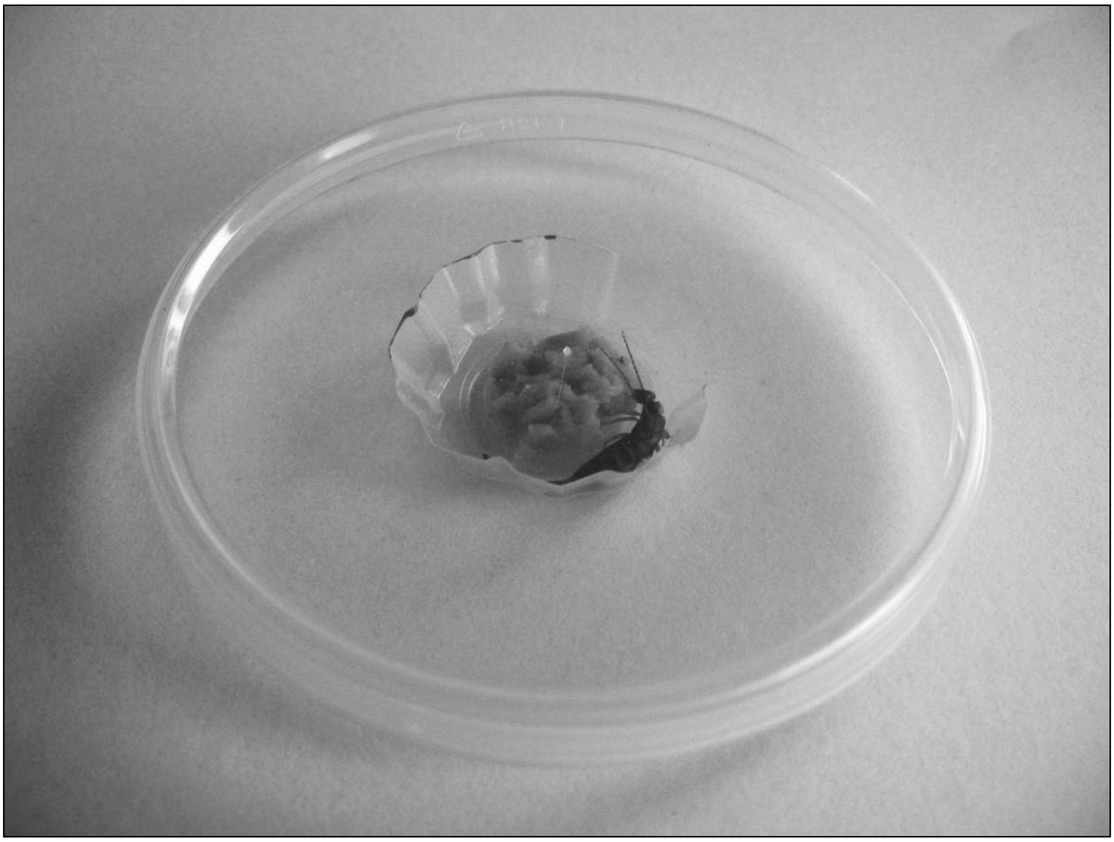

Figure 3. The experimental set-up used in palatability tests with earwig consumers.

Results and discussion of the earwig experiment

There was no significant difference in the times that earwigs fed on the mixtures containing non-melanic and melanic *H. axyridis* (two-tailed paired *t*-test: t=1.4, 17 df., P=0.18; Fig. 4). Results trended towards a shorter feeding period on the melanic rather than the non-melanic mixture (i.e. the opposite way from that predicted by Bezzarides *et al.* (2007); Fig. 4).

As pointed out above, the results, which suggest no difference in the defences of the two morphs, must be considered very tentative. There are a number of reasons for this. Only one mixture for each morph was tested (i.e. individual replicates were not independent), and it is risky to generalise from these two mixtures to the two morphs overall, without the testing of further similar mixtures. Although experiments comparing interspecific differences in chemical defence have used the same approach (e.g. Marples, 1993b), an intraspecific difference may be of a smaller magnitude, making the method less robust and variation more difficult to detect. Differences in individual ladybirds, such as size, previous nutritional experience, reproductive state and whether the ladybirds have recently used their chemical defences could affect the overall properties of the mixture. The ideal experiment would, in addition to using several independent mixtures, also include a control, unadulterated mixture to ensure that the two mixtures were truly less attractive to the earwigs (rather than relying on prior testing), and consequently would have a much larger matched sample size to account for multiple comparisons. It would be desirable to test males as well as females.

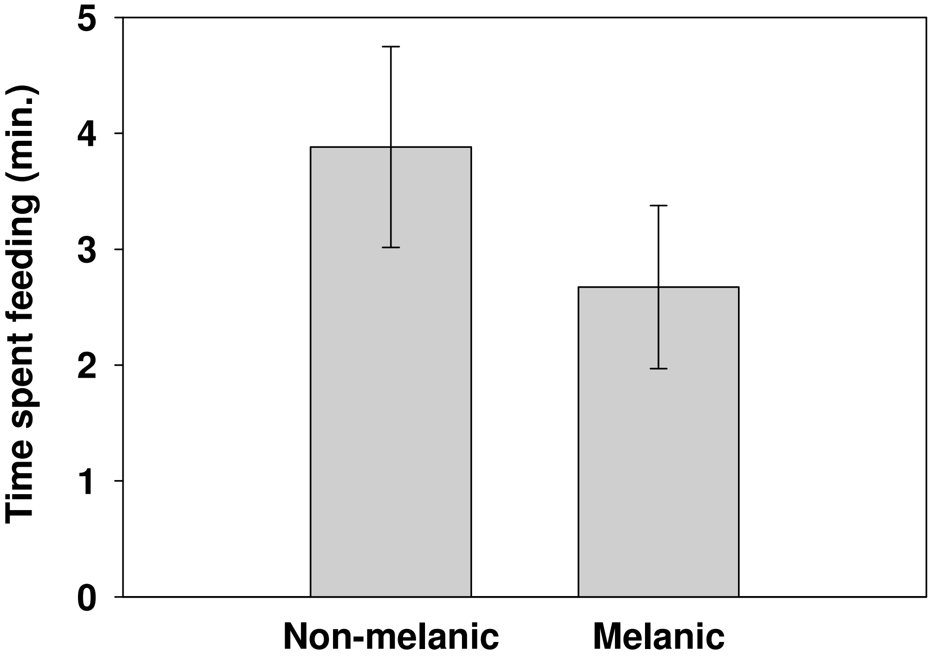

Figure 4. Results (presented as means ± S.E.) of palatability experiments with earwig consumers and food mixture containing non-melanic or melanic *Harmonia axyridis*. *N*=18 matched trials.

Concluding discussion

Neither of the experiments described here, with spiders or earwigs, indicate that non-melanic *H. axyridis* are better defended than melanics, as suggested by Bezzarides *et al.* (2007). It would be nonetheless premature to conclude that the difference does not exist on the basis of these experiments alone. The experiments with spiders are relatively uninformative due to the generally high level of acceptability of ladybirds as prey, while experiments with earwigs are of too small a scale to be unequivocal. It is noteworthy, however, that in both cases, though in neither significantly, melanics appeared, if anything, to be slightly less acceptable than non-melanics, a trend unsupportive of non-melanics being the better defended of the two forms.

The experiments described here were designed as bioassays of the strength of chemical defence possessed by different forms of *H. axyridis*. The consumers involved are not the sort of visually-hunting predators that would be likely to use colour pattern as a potential signal of chemical repellency or toxicity: such predators are most likely to be vertebrates. That does not mean that putative differences in chemical investment would not result in differential losses of the forms to invertebrate natural enemies. However, such losses would not be a direct consequence of morph type; rather selective pressure on colour pattern would act through predator selection of more weakly chemically defended individuals. By contrast, visual predators could choose prey directly on the basis of their colour pattern, as an indicator of chemical strength.

Two particular points should be emphasized about these studies in relation to that of Bezzarides *et al.* (2007). The first is that melanics were not examined in Bezzarides *et al.*'s work: the idea that melanics are less well defended arises entirely from their argument that individuals with greater areas of orange on their elytra are better defended. It remains a possibility that the positive correlation between the proportion of the elytra that is orange and harmonine concentration is restricted to non-melanics and does not occur universally across all morphs of *H. axyridis*.

The second point is that Bezzarides *et al.* only studied harmonine concentration and harmonine is only one component of *H. axyridis* chemical defence. The ladybird also utilises another alkaloid, 3-hydroxypiperidine-2-one (Alam *et al.*, 2002), and foul-smelling methoxypyrazines, which serve as an olfactory equivalent of warning colouration (Cai *et al.*, 2007). Interestingly, there is some evidence of a positive correlation between methoxypyrazine concentration and orange intensity (Cai *et al.*, 2007), although orange intensity was unrelated to harmonine content in Bezzarides *et al.*'s study. The occurrence of two alkaloids rather than one, as well as the methoxypyrazines, complicates the situation: specifically, it makes it less likely that harmonine concentration and acceptibility or suitability for natural enemies will be well correlated. It may be that while harmonine concentration is correlated with the proportion of the elytra that is orange, 3-hydroxypiperidine-2-one concentration and/or total alkaloid concentration is not. The relative importance of the two alkaloids in conferring chemical protection remains unknown and could differ; this would also increase the likelihood that the effects of two alkaloids in combination might outweigh intraspecific differences in harmonine content. Similarly, if predators initially respond to the pyrazines through olfaction, this could override the effects of differences in alkaloid concentration on prey selection. Overall, it should be borne in mind that consumer responses when confronted with whole prey (or food derived thereof), will be a function of all defensive chemicals combined, and not just harmonine.

Majerus (1994) points out that the factors maintaining colour pattern polymorphisms in ladybirds are complex, variable and interactive and that there is a need for a more rigorous technique in dissecting them out. This conclusion, applicable across all ladybird colour pattern polymorphisms, is particularly pertinent at this early stage to work on intraspecific variation in chemical defence and colour pattern. More analytical work is necessary, in addition to further studies of palatability and toxicity. In *H. axyridis* this work should include the full range of defensive compounds, as well as colour pattern forms. Only then will we understand what is potentially a complicated, multidimensional relationship, and can place it in its correct ecological context, alongside other factors already known to play a role in maintaining ladybird colour pattern variation.

Dedication

This paper was given in memory of the late Professor Mike Majerus (1954-2009), the author's former Ph.D. supervisor. Through his own research and his influence on students and colleagues, he made a significant contribution to our views on colour pattern polymorphism in ladybirds and other insects, as well as in many other areas of ladybird biology and entomology. His enthusiasm for ladybirds went far beyond the more typical, impact factor-driven university research, making his 1994 New Naturalist book *Ladybirds* and his numerous entomological notes some of the most valuable resources available on ladybird natural history. His influence is evident in the work outlined here, not only in the choice of subject matter, but in the fact that the consumers tested were chosen from a careful reading of his *Ladybirds* book chapter on ladybird death and natural enemies.

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The establishment and rapid spread of an alien invasive lady beetle: *Harmonia axyridis* (Coleoptera: Coccinellidae) in southern Africa, 2001–2009

Riaan Stals
South African National Collection of Insects (SANC), Biosystematic Division, ARC-Plant Protection Research Institute, Private Bag X134, Queenswood, Pretoria, 0121 South Africa: Corresponding author: StalsR@arc.agric.za

Abstract: That *Harmonia axyridis* (Coleoptera: Coccinellidae), a grievous alien invasive species, has established in southern Africa and is spreading though the region is presently not well known outside South Africa. The first known record for the region is a capture from 2001. Establishment is hypothesised to have taken place in the south-western part of the Western Cape Province. The geographic spread of the insect through southern Africa has been recorded since 2006 and retrospectively, with information largely obtained through citizen science. Until the end of 2009, the beetle has spread widely through the more temperate southern and higher-lying eastern and east-central parts of South Africa, and has also been recorded from Lesotho. In 2009 it has additionally been recorded from localities in the hotter, drier interior of South Africa for the first time. The invader has been found in a large variety of natural and transformed landscapes and habitats. The ecological effects it may exert may ultimately be unknowable because of the absence of baseline information on coccinellid community ecology in southern Africa.

Key words: *Harmonia axyridis*, South Africa, Lesotho, alien invasive species, range expansion, citizen science

Introduction

The current invasion of Europe, the Americas and southern Africa by the alien ‘harlequin’ lady beetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), is accompanied by much attention from biologists, interested laypeople and the popular media. An enormous volume of literature has emerged from contemporary research on the ‘harlequin phenomenon’, burgeoning almost exclusively from the Northern Hemisphere. The ecobiology and use in biological control of the species were, among others, reviewed by Koch (2003) and Pervez & Omkar (2006). Its invasive ecology and implications thereof were reviewed by a large number of mostly European researchers (Babendreier, 2007, Roy & Wajnberg, 2008). *Harmonia axyridis*, native to east Asia, has in recent years emerged as a significant invader on several continents, being eurytopic, broadly polyphagous, very dispersive and phenotypically and ecologically highly plastic. It potentially can cause complex ecological effects involving changes in invaded communities, besides impacting on agriculture and human health, and being a noteworthy household nuisance.

From the Southern Hemisphere, the first discussion of the ‘harlequin invasion’ of South America was that of Koch *et al.* (2006), whereas Stals & Prinsloo (2007) announced the arrival and establishment of this invader in South Africa. It appears that *H. axyridis* has not (yet) been found in the field in Australia or New Zealand, although it has been intercepted at ports of entry to Australia at least twice (Carvan, 2009; Government of Western Australia, 2008).

Whereas impressive monitoring schemes, accompanied by significant research effort, are in place in several countries in the Northern Hemisphere (e.g. Roy & Wajnberg, 2008), the limited human and other resources for entomology in southern Africa have as yet precluded structured surveying for *H. axyridis* in this region. The early detection of this apparently inadvertent introduction to South Africa presented a rare prospect to study a conspicuous biological invasion virtually from its incipience. There has, somehow, not been many takers of this opportunity. I have been keeping a record of the geographic spread of this alien invasive through southern Africa since 2006; some initial results of this study are presented here.

Methods

Citizen science (e.g. Silvertown, 2009) was employed to generate most of the information presented here. Records of the presence of *Harmonia axyridis* were collected fortuitously and opportunistically. The first records, and some later records, were those of specimens submitted to the identification service of the South African National Collection of Insects (SANC), Pretoria.

Since March 2008, a continuing series of articles about the 'harlequin phenomenon' is being published in *Plant Protection News*, the quarterly newsletter of the ARC-Plant Protection Research Institute, which includes the SANC. Without fail, each article leads to a renewed volley of media attention and a concomitant surge of contributions from the general public, who were invited to provide samples and records of observations of *H. axyridis*. Furthermore, public interest is sustained through participation in radio broadcasts, public lectures, articles in the popular press, media interviews, and the furnishing of the media with information and photographs.

No records were accepted without vouchers, which could either have been real specimens or photographs of adult, larval or pupal *H. axyridis* from an unambiguous locality. All vouchers are deposited in SANC. Submissions were accepted through email, ordinary mail and personal delivery or collection.

Additionally, since 2006, I have inspected almost all the public insect specimen collections in South Africa for the presence of *H. axyridis* specimens, and enquiries about South African *H. axyridis* specimens were sent to selected taxonomists of Coccinellidae and the curators of selected museum collections in other countries after the discovery of the insect in South Africa.

In this contribution, the geographic range expansion over time is presented on maps of southern Africa at the coarse scale of half-degree squares (approaching 50km-squares at South Africa's latitudes).

Results and discussion

Establishment of **Harmonia axyridis** *in South Africa, and apparent initial lag in spread*

The first South African specimens (adults, eggs and larvae) of *Harmonia axyridis* to become known as such were collected by Dr Goddy Prinsloo, researcher of the ARC-Small Grains Institute, Bethlehem, South Africa, in October 2006 in a wheat field experiment near the town of Riviersonderend in the Western Cape Province of South Africa; at that time a population of the species was clearly established and reproducing at this locality (Fig. 1: southernmost black square). *Harmonia axyridis* had at this locality already been observed, by Goddy Prinsloo, during September/October 2004 (Stals & Prinsloo, 2007), but specimens were only submitted to the SANC identification service two years later. Upon enquiry from museums in 2006, a single *H. axyridis* specimen that had previously been collected in South Africa came to light. This singleton, lodged in the Natural History Museum of the Humboldt University, Berlin, Germany, was captured in December 2002 at a locality 40km northwest of Riviersonderend (Fig. 1). Stals & Prinsloo (2007) presented these records as the earliest known South African records for *H. axyridis*.

In September 2009, I discovered older South African specimens of the species—at the time either misidentified or unidentified—in the Iziko South African Museum (SAMC), Cape Town, and the insect collection of the University of Stellenbosch. All those specimens were collected in and around Cape Town and Stellenbosch (both Western Cape Province) during June and July 2001, during at least four separate collecting events (Fig. 1: westernmost black rectangle). One series of these (adult beetles in SAMC) carries the collecting data that adults, larvae and pupae were present, indicating an established adventive population. These presently constitute the earliest record of the presence of *H. axyridis* in southern Africa.

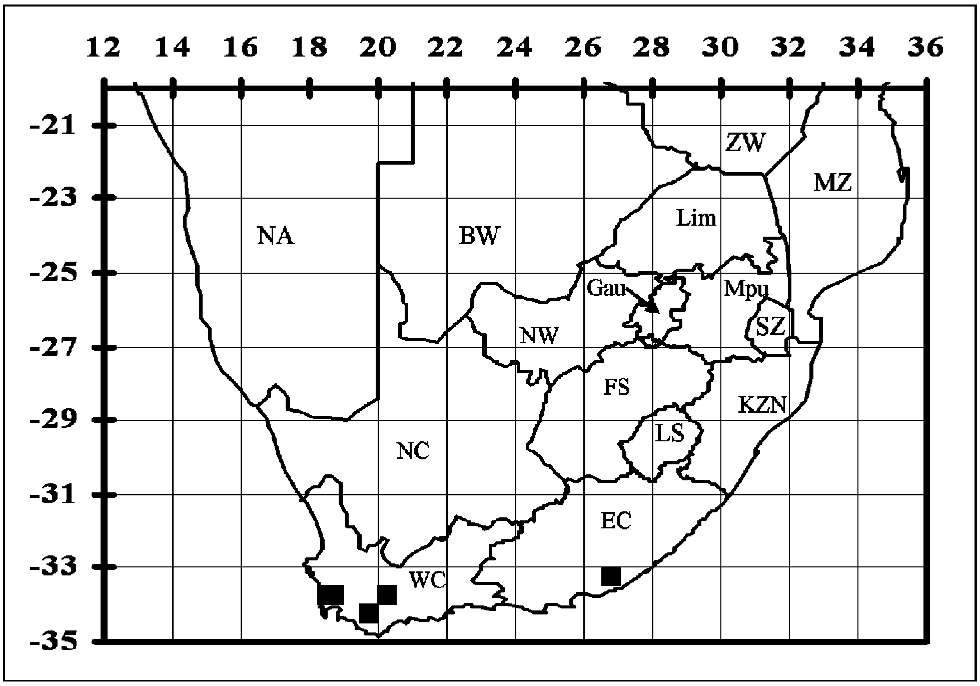

Figure 1. Recorded distribution of *Harmonia axyridis* in southern Africa, up to September 2007 (the end of Winter). Provinces of South Africa: EC, Eastern Cape; FS, Free State; Gau, Gauteng; KZN, KwaZulu-Natal; Lim, Limpopo; Mpu, Mpumalanga; NC, Northern Cape; NW, North-West; WC, Western Cape. Other countries: BW, Botswana; LS, Lesotho; MZ, Mozambique; NA, Namibia; SZ, Swaziland; ZW, Zimbabwe.

The mechanism and source of this invasion are unknown. Currently I hypothesise that the initial establishment of *H. axyridis* in South Africa was in the south-western Western Cape Province, where, in close proximity to each other, there are two major seaports (Cape Town and Saldanha), an international airport and the hub of three major national highway systems, with large volumes of foreign and domestic travel and trade; a large and fairly densely populated metropolitan area; and large areas of intensive agriculture. This area, within the Cape Floral Kingdom, happens to coincide with a biodiversity hotspot in the sense of Myers *et al.* (2000), as well as being a hotspot of alien invasive species (e.g. Joubert, 2009).

The only other known *H. axyridis* specimens collected in southern Africa until the end of the (austral) winter of 2007 were found in a horticultural field experiment close to Grahamstown in the Eastern Cape Province, by a graduate student at Rhodes University, Grahamstown, in October 2006 and March 2007, (Stals & Prinsloo, 2007) (Fig. 1: easternmost black square). Whereas the Riviersonderend and Cape Town/Stellenbosch localities are less than 150km apart, the Eastern Cape specimens were collected more than 600km further to the east. From October 2006 to September 2007, no localities, newly recorded for this species in southern Africa, are known. Although the available data are insufficient for a confident assumption, it seems as if the species had, after probable arrival and establishment in or before 2001, not spread outside the south-western part of the Western Cape Province for between five and six years.

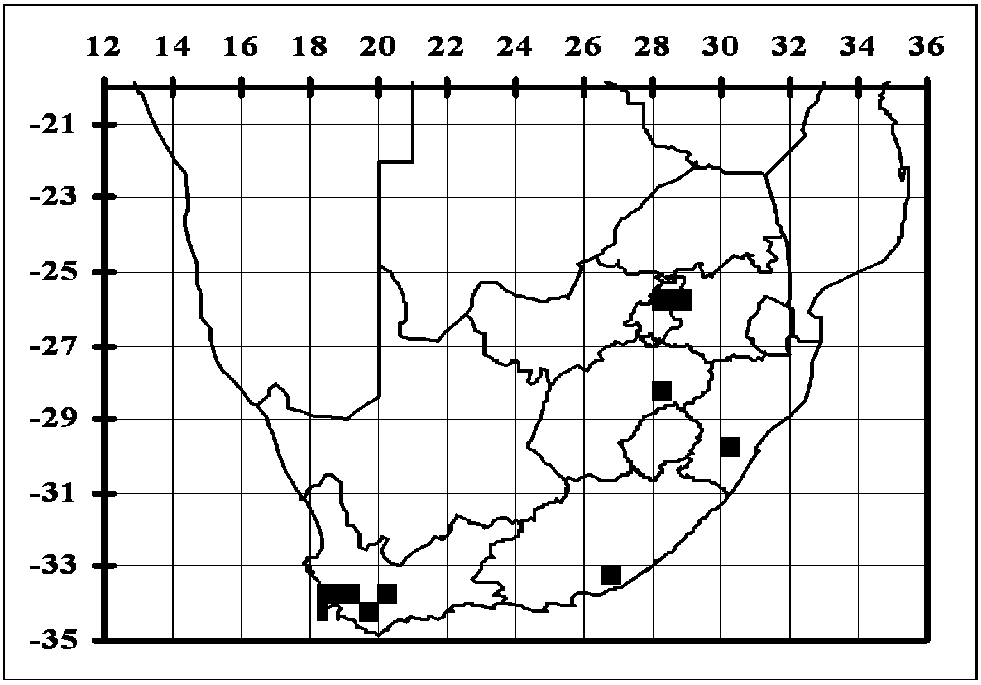

Figure 2. Recorded distribution of *Harmonia axyridis* in southern Africa, cumulative until March 2008 (the end of Summer). Territories as in Fig. 1.

The spread of **Harmonia axyridis** *through southern Africa*

The journal issue in which the arrival of *H. axyridis* in South Africa was announced (Stals & Prinsloo, 2007) was published in August 2007. That elicited reaction from a couple of researchers from the Agricultural Research Council and two universities in South Africa during the subsequent period up to March 2008. Besides increasing the extent of the known distribution of the ‘harlequin’ in the south-western Cape (Fig. 2), new records thus obtained, and one serendipitously collected by myself, included the first records for the provinces KwaZulu-Natal, Free State and Gauteng (Fig. 2). The distances between the (clusters of) newly recorded localities were very large (roughly 250–550km), and it is unknown whether, but perhaps likely that, undetected populations of *H. axyridis* were at that time also present in the intervening ‘gaps’.

The first article about the South African ‘harlequin invasion’ in *Plant Protection News* was published in March 2008, sparking instantaneous media attention which, in turn and unexpectedly, triggered a little avalanche of reports of *H. axyridis* from the general public. The number of quarter-degree squares (each corresponding to *c.* 625km^2 at 28° latitude) with at least one verified report of *H. axyridis* being present increased from eleven in March 2008 to 47 in December 2008. This is, however, an incomplete reflection of the records collected, as numerous “duplicate” reports were received from a number of quarter-degree squares. Such localities tended to fall within the great conurbations of Gauteng and the Cape Peninsula (south-westernmost Western Cape Province) (Figs 3, 4), a trend which probably correlates better with the availability of digital cameras and access to email than with any biologically meaningful parameter.

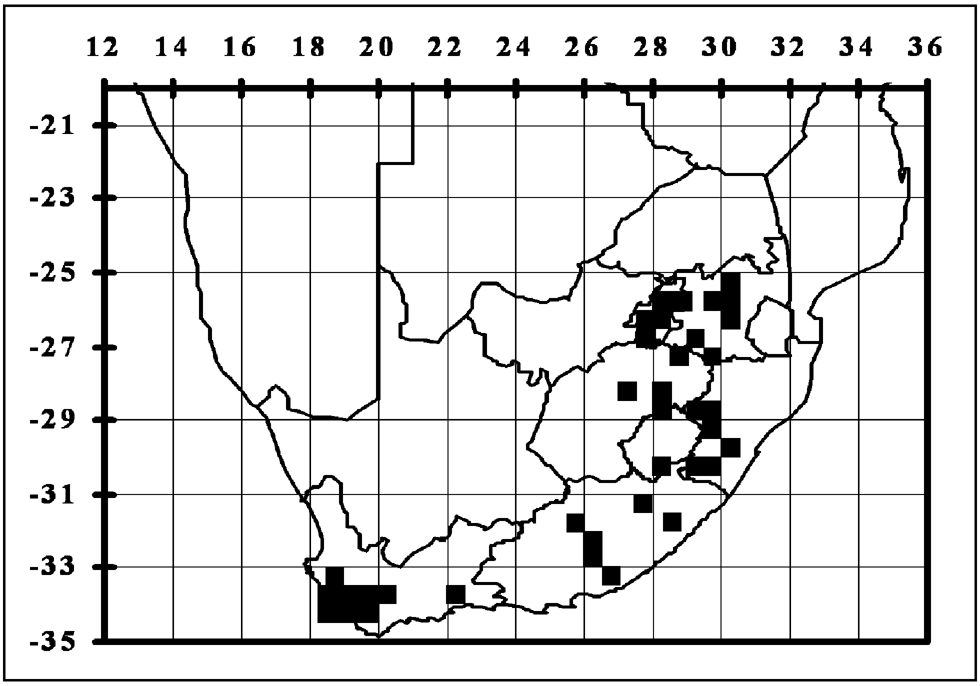

Figure 3. Recorded distribution of *Harmonia axyridis* in southern Africa, cumulative until the end of 2008. Territories as in Fig. 1.

Localities from which *H. axyridis* were newly reported in 2008 were distributed predominantly in the eastern and east-central, more mesic and temperate areas of South Africa (Fig. 3) and mostly fell in the Grassland Biome. In June 2008 the first ‘harlequins’ were recorded from Lesotho (Fig. 3).

During 2009, the presence of *H. axyridis* was verified in an additional 18 quarter-degree squares (Fig. 4), besides even more records from quarter-degree squares previously recorded. The apparent increase in the invader’s distribution was evident in the south-western and southern Cape (both Western and Eastern Cape Provinces) and in the higher-lying eastern and central grasslands (in the provinces Eastern Cape, KwaZulu-Natal, Free State, Gauteng and Mpumalanga) (Fig. 4). Some of the ‘gaps on the map’ from the previous years were beginning to be filled (Figs 3, 4).

By the end of 2009 (Fig. 4), *H. axyridis* had been reported from six of the seven biomes (following Low & Rebelo, 1996) of South Africa. The insect has been found at sea level and low altitudes in the south-western and southern Cape and at altitudes in excess of 1800m in the Drakensberg mountains of KwaZulu-Natal and Lesotho. It is noticeably absent from low elevations in KwaZulu-Natal (Fig. 4), where the low-lying coastal belt is hot and humid.

Significantly, the first records from localities in the dry interior were received and verified during 2009 (Fig. 4). Those from the Northern Cape Province fall in the Nama Karoo Biome, which is a generally hot and dry semidesert area, and in the North-West Province in hot and dry savanna, approaching Kalahari Thornveld. Also in 2009, the first records were received from hot, low-lying savanna areas, at 500-700m above sea level and below the central plateau of southern Africa. The northernmost southern African record to date, near the town of Tzaneen, is from barely 40 km south of the Tropic of Capricorn.

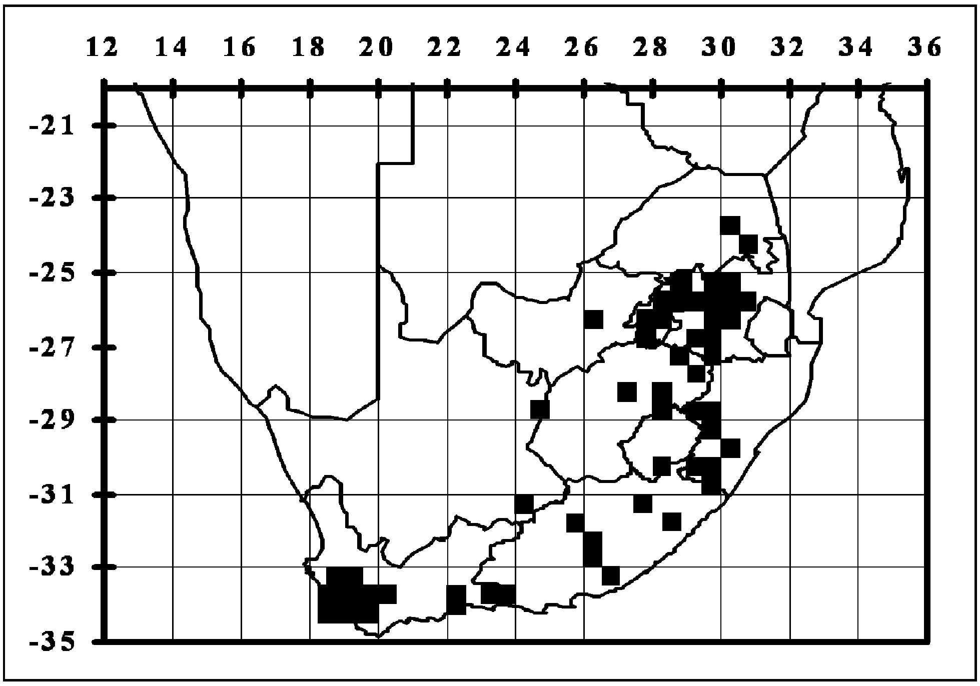

Figure 4. Recorded distribution of *Harmonia axyridis* in southern Africa, cumulative until the end of 2009. Territories as in Fig. 1.

Most records contributed by the public were from an urban or suburban environment, with numerous contributors reporting invasions of buildings and large aggregations of *H. axyridis* during the drier and cooler months in the KwaZulu-Natal, Mpumalanga and Gauteng provinces. Besides being recorded from numerous urbanised areas, *H. axyridis* has also been found in various agricultural settings, including plantings of field and vegetable crops, vineyards, deciduous and subtropical fruit orchards, and plantation forestry. It is likely to have established populations in all of these. It appears that very large populations are present throughout the important winelands of the Western Cape Province, leading to justifiable concern in the wine industry (e.g. Allsopp, 2008). *Harmonia axyridis* has, additionally, also been found in various natural field settings, importantly including habitats of unspoilt fynbos (Cape maquis) and seemingly pristine grassland. Apparently established populations are already known from a number of nature conservation areas, including national and transfrontier parks.

All southern African specimens of *H. axyridis* examined to date belong to various subforms of the colour form *succinea*, with only one series of specimens (Western Cape Province) hitherto examined displaying some melanic suffusion on the dorsum.

Concluding remarks

Despite the obvious and significant shortcomings of this study, it is turning out to be one of the more comprehensive and detailed documentations of a southern African invasion by an alien animal species. An opposite case in point, sadly more typical, is that of *Hippodamia variegata* (Goeze), similarly an aphidophagous Palaearctic lady beetle that by unknown means had reached the region, established and spread. The documentation of its arrival and establishment in South Africa is limited to an *en passant* comment in a somewhat obscure source (Aalbersberg *et al.*, 1984). Today, *H. variegata* is ubiquitous in southern Africa, locally abundant, and presently not appreciated to be an alien invader by probably the majority of local field entomologists (Stals, unpublished). The ecological impact of its southern African invasion is undocumented and unknowable.

It is widely accepted that the tracking of introduced species is an important component of invasion biology research (e.g. Strayer *et al.*, 2006). This is done, albeit somewhat rudimentarily, in the case of *H. axyridis* in southern Africa. Besides that, this species's ecological impact in the region may still prove to be unknowable in the light of the utter dearth of published studies dealing with coccinellid community ecology in Africa. Since there is a great lack of baseline information, we are ignorant about the *status quo*. Hence we may, in time, find that *Harmonia axyridis* has become as much part of our fauna as *Hippodamia variegata* had previously become, and be equally ignorant about any changes in ecological communities it may have been the cause of.

Acknowledgements

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Temperature dependent development of *Harmonia axyridis* Pallas (Col.: Coccinellidae) on two prey: *Aphis fabae* Scopoli and *Dysaphis crataegi* (Kaltenbach) (Hem.: Aphididae)

George J. Stathas[1], Dimitris Kontodimas[2], Filitsa Karamaouna[2] & Stavros Kampouris[1]
[1]Technological Educational Institute of Kalamata, 24100-Antikalamos, Greece; [2]Benaki Phytopathological Institute, 8 St. Delta str, 14561-Kifissia, Greece
Corresponding author: georgestathas@hotmail.com

Abstract: Development of the predator *Harmonia axyridis* was studied at four constant temperatures (15°, 20°, 25° and 30°C) on two prey, *Aphis fabae* and *Dysaphis crataegi,* in laboratory conditions. Total developmental time of the predator at 15°C and 30°C was shorter on *D. crataegi* (76.7 and 16.6 days, respectively) than on *A. fabae* (90.2 and 18.3 days respectively) but did not differ significantly between the species at 20°C (36.8-38.9 days) or 25°C (24.0-24.9 days). The thermal constant (K) of development of *H. axyridis* from egg to adult was 258.2 day-degrees above a lower developmental threshold of 11.2°C, on *A. fabae,* and 243.6 day-degrees above a lower developmental threshold of 10.8°C, on *D. crataegi.*

Key words: aphids, predator, pumpkin, rearing, thermal threshold

Introduction

The predator *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) was introduced in Greece from France for the control of aphids in central and southern Greece from 1994 to 1999 where it was released at various crop and ornamental plants production sites (Katsoyannos *et al.*, 1997; Kontodimas *et al.*, 2008a; Kontodimas *et al.*, 2008b). Mass rearing of *H. axyridis* for the purpose of releases was performed on two aphid species, *Aphis fabae* Scopoli and *Dysaphis crataegi* (Kaltenbach) (Hem.: Aphididae) under controlled laboratory conditions in the insectary of Benaki Phytopathological Institute, Athens (Kontodimas *et al.*, 2008a, b). *Harmonia axyridis* proved to be an important biological control agent during the release seasons but follow up surveys at the release sites during 1994-2007 indicated the inability of the predator to establish in Greece, as reported in Portugal and Spain (Brown *et al.,* 2008). *Harmonia axyridis* imposed no adverse effects on the native coccinellid fauna in Greece (Kontodimas *et al.*, 2008a, b).

The developmental rate in insects depends on the environmental temperature to which they are exposed, therefore the lower developmental threshold (t) and the day-degree requirements for their development (thermal constant –K) may be useful indicators of an insect's potential distribution (Messenger, 1959). The relationship between the development rate and temperature is usually curvilinear (Davidson, 1944; Sharpe & DeMichele, 1977; Mills, 1981). However, within the limits of a certain temperature range, this relationship is linear allowing an estimate of the lower developmental threshold and thermal constant using the linear model by Campbell *et al.* (1974). Nevertheless, in the case of *H. axyridis*, both prey species and the host-plant can affect its larval developmental time (Hukusima & Kamei, 1970).

This project aims at estimating the lower developmental threshold (t) and thermal constant (K) of the predator *H. axyridis* when it preys on the aphids *A. fabae* or *D. crataegi*.

Material and methods

Insect rearing

Individuals of *H. axyridis* used in the experiments originated from the colony imported from France and established at Benaki Phytopathological Institute in 1994 (Katsoyannos *et al.,* 1997). *Harmonia axyridis* was reared on the aphids *A. fabae* and *D. crataegi*. *Aphis fabae* was reared on seedlings of *Vicia faba* L. (Leguminosae) at 22°C, R.H. 60±5% and 16/8 L/D while *D. crataegi* was reared on pumpkins *Cucurbita moschata* (Duchesne ex Lam.) (Cucurbitaceae) at 25°C, R.H. 60±5% and 16/8 L/D.

Experiments for the temperature effect on developmental duration of all developmental stages of *H. axyridis* started with eggs of mated females, which were reared on either aphid species in cylindrical Plexiglas cages (30cm in diameter, 50cm in length) (Iperti & Burn, 1969) at four constant temperatures, 15°±0.5°, 20°±0.5°, 25°±0.5° and 30°±0.5°C, R.H. 60±5% and photoperiod 16/8 L/D. The developmental duration of egg, larval and pupal stage as well as the preoviposition period was measured for individuals reared on *A. fabae* or *D. crataegi* in Petri dishes (9cm diameter), at the aforementioned temperatures. A total of 25 Petri dishes with single individuals were used for each aphid species-temperature combination. Measurements were recorded every 12 hours.

After measurement, individuals were supplied with up to 100 aphids per Petri dish to secure food abundance and avoid the effects of lack of food on the duration of development, as it is reported for coccinellids in many studies (Hodek & Honěk, 1996). Specifically for *H. axyridis*, according to Hukusima & Ohwaki (1972), decrease of the food rates increased developmental time and slowed down larval growth. The offered quantity of food in the present study (up to 100, per individual, twice per day) is known to be the maximum daily prey consumption of larvae and adults by *H. axyridis* when fed on *A. fabae* and *D. crataegi* (Stathas *et. al.*, unpublished data).

Data analysis

Estimation of lower developmental threshold and thermal constant was based on the linear model by Campbell *et al.* (1974) using the statistical program JMP (Sall *et al.,* 2001). Data on developmental duration were subjected to Analysis of Variance with $\alpha = 0.05$ and means were separated using the Tukey – Kramer HSD Test (Sokal & Rohlf, 1995) using JMP.

Results and discussion

The linear model (Campbell *et al.,* 1974) seems to describe well the developmental rate of *H. axyridis*, fed on either aphid species, within the temperature regime of the study (15-30°C) (coefficient of determination for developmental rate from egg to adult: $R^2 = 0.9993$ on *A. fabae* and $R^2 = 0.9987$ on *D. crataegi*).

The duration of life cycle of the predator at 15°C and 30°C was shorter on *D. crataegi* (76.7 and 16.6 days, respectively) than on *A. fabae* (90.2 and 18.3 days, respectively) but it did not differ significantly between the species at 20°C (36.8-38.9 days) or 25°C (24.0-24.9 days). Hence, if 30°C is proved to be the most favourable temperature for mass rearing of the predator in the insectary, due to shorter development, *D. crataegi* could be a better diet compared to *A. fabae*. However, the effect of temperature on other biological parameters of the predator, such as fecundity, should be considered, too.

The thermal constant (K) of development (egg to adult) of *H. axyridis* was 258.2 degree days above a lower developmental threshold of 11.2°C on *A. fabae* and 243.6 degree days above a lower developmental threshold of 10.8°C on *D. crataegi*. This discrepancy in the estimates of thermal constant could be attributed to the influence of differences in developmental threshold and/or food quality between the aphid species or in the environmental conditions (i.e. relative humidity) of the experiments (Hukusima & Ohwaki 1972; Stathas, 2000). In comparison with these results, development of the predator from egg to adult was shown to require 267.3 degree days above a lower developmental threshold of 11.2°C in the United States (LaMana & Miller, 1998) and 231.3 degree days above a lower developmental threshold of 10.5°C in France (Schanderl *et al*., 1985). Moreover, studies on the effect of other aphid prey, *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae) and *Rhopaloshiphum maidis* (Fitch) (Hemiptera: Aphididae), on the development of seven coccinellid species have shown that the total developmental length increased when the food was unsuitable (Smith 1965a, b, c; Obrycki & Orr, 1990).

Overall, our results suggest similar thermal requirements for the development of *H. axyridis* on the particular prey, compared to data on other aphid species used as prey, in the literature (LaMana & Miller, 1998; Schanderl *et al*., 1985). As *H. axyridis* seem to meet these thermal requirements for development in Greece and it is known to be able to survive very low temperatures during winter (lower lethal temperature was estimated at -16°C in Japan by Watanabe, 2002) and to migrate to overwintering sites (in Asia and Ohio by Liu & Qin, 1989; Sakurai *et al.,* 1993; Kidd *et al.,* 1995; LaMana & Miller, 1996), further investigation is needed in order to explore the environmental reasons for the difficulties of the predator to establish in Greece.

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Entomopathogenic fungi found in field populations of the harlequin ladybird, *Harmonia axyridis*

Tove Steenberg*[1] & Susanne Harding[2]
[1]Department of Integrated Pest Management, University of Aarhus, Skovbrynet 14, DK-2800 Kgs. Lyngby, Denmark; [2]Department of Agriculture and Ecology, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark.
Corresponding author: Tove.Steenberg@agrsci.dk

Abstract: A survey of natural enemies in larvae (including prepupae), pupae and adults of the harlequin ladybird, *Harmonia axyridis*, showed that several species of entomopathogenic fungi could be isolated from samples of the three life stages collected at different times of year. In 2007-2008 *Isaria farinosa* was the most prevalent species in larvae and pupae. In contrast, in the autumn of 2009 *Beauveria bassiana* was the dominant species in larvae, prepupae and pupae. The prevalence of fungus infection varied greatly between locations, life stages and time of year. We also report the finding of the parasitic fungus *Hesperomyces virescens* from an adult *H. axyridis* collected in Germany.

Key words: entomopathogenic fungi, *Harmonia axyridis*, *Beauveria bassiana*, *Isaria farinosa*, *Lecanicillium* spp., *Hesperomyces virescens,* mortality, natural enemy

Introduction

Harmonia axyridis was first recorded in Denmark in 2006 and was well established by autumn 2007, when large numbers of immature stages and adults were recorded (Steenberg & Harding, 2008). Since then we have studied the adaptation of native natural enemies to *H. axyridis*. The literature on naturally occurring entomopathogenic fungi in coccinellids is scarce and mostly refers to overwintering adults (e.g. Iperti, 1964; Lipa et al., 1975; Ceryngier, 2000). *Harmonia axyridis* adults have been reported to be only marginally susceptible to infection by the hypocrealean fungus *Beauveria bassiana* in laboratory experiments (Cottrell and Shapiro-Ilan, 2003, 2008; Roy et al., 2008), and the only previous report of fungus infection in field populations of *H. axyridis* is from Russia in the natural distribution area, where the impact of *Beauveria* sp. on adult *H. axyridis* was minimal (Kuznetsov, 1997).

The lack of information on natural enemies of *H. axyridis* is to a great extent due to a lack of field studies. Therefore we initiated a survey of the occurrence of entomopathogenic fungi and other natural enemies immediately after the establishment of *H. axyridis* in Denmark. Here we report data on fungal pathogens.

Material and methods

18 samples of *H. axyridis* were taken in Copenhagen from late autumn 2007 to June 2008. In late autumn and early winter 2007 larvae and pupae were collected from tree trunks, statues and fences. These specimens were mostly moribund or dead at the time of collection, but a

small number of larvae and pupae was alive and was also included in the study. During the winter 2007/2008 overwintering adults were sampled and kept in a climate cabinet at 6ºC, 65% relative humidity and no light after collection. By March, mortality was recorded. In May-June 2008 pupae (almost exclusively alive at the time of collection) were taken from leaves and trunks of *Acer* and *Tilia*. In total, 3221 specimens were collected in 2007-2008. Dead individuals were placed on moist filter paper to facilitate sporulation by fungal pathogens.

Additional samples of last instar larvae (L4), prepupae and pupae (47 samples in total) were collected from 11 sites in Copenhagen from August to October 2009. A total of 250 L4, 250 prepupae and 1657 pupae were sampled. In contrast to the collections in late autumn 2007, most larvae and pupae were alive at the time of collection. For prepupae it was not possible to ascertain whether they were dead or alive. Adult *H. axyridis* (272 specimens) were collected in August-September. Dead or moribund individuals were incubated as described above.

Results and discussion

In 2007-2008 fungi were recorded from 15 of 18 samples; 109 fungus killed specimens were found. *Isaria farinosa*, *Beauveria bassiana* and at least 2 species of *Lecanicillium* were recorded from *H. axyridis* (Fig. 1, Fig. 2). The dominant species was *Isaria farinosa* that infected 73 of the fungus-killed specimens. All fungus species have also been isolated from native coccinellids in Denmark (Steenberg & Harding, 2009). The widespread occurrence of entomopathogenic fungi in populations of *H. axyridis* was confirmed in 2009, where fungus-infected specimens were found in 23 of 47 samples. However, in 2009 *B. bassiana* was the most commonly found fungus: 72 of 116 fungus-killed specimens were infected by this species.

Figure 1. A: *H. axyridis* pupa infected by two species of entomopathogenic fungi: *Lecanicillium* sp. (arrow) and *Beauveria bassiana.* B: *H. axyridis* killed by *Beauveria bassiana.* The beetle was found moribund on a tree trunk, 20 January 2008. C: Coremia of *Isaria farinosa* protruding from a pupa. Photos: Tove Steenberg.

Fungus prevalence in overwintering adults in 2007-2008 ranged from 1.9 to 17.9%. Pupae were infected in slightly lower proportions (0.5 to 3.8% infected in early summer 2008, 1.0 to 11.9% infected in late summer 2009). In contrast to the winter mortality observed for adults in the winter 2007-2008, no fungus infection was found in adults collected in late summer 2009. Winter mortality levels attributed to fungal pathogens in adult *H. axyridis* reached levels comparable to those published for other coccinellid species (Iperti, 1964; Lipa et al., 1975; Ceryngier, 2000). The finding of fungus-infected adult *H. axyridis* is interesting because other studies have failed to find fungus infection in overwintering adults of this species (Cottrell & Shapiro-Ilan, 2003). In addition, laboratory infection experiments have shown that adult *H. axyridis* are significantly less susceptible to infection by *B. bassiana* than the native species *Coccinella septempunctata* and *Adalia bipunctata* (Roy et al., 2008; Steenberg, unpublished data). At present, it cannot be excluded that the fungus infection observed in overwintering adults may be the result of hosts being stressed and more prone to succumb to infection.

Laboratory infection experiments with larvae of *H. axyridis* indicate that larvae are significantly more susceptible to fungus infection than adults (Steenberg, unpublished; Steenberg & Harding, 2009). The field collections in autumn 2007 and 2009 confirm that fungus prevalence in larvae and pupae in some cases can be high. In 2007, 28% of larvae collected live in November were fungus-infected (N=25) as were 27% of live pupae (N=15). In 2009, samples taken from a location with 3 *Acer* trees on 13 October showed that 44% of last-instar larvae (N=9), 82% of prepupae (N=38) and 10% of pupae (N=133) were infected by entomopathogenic fungi. We suggest that the high fungus prevalence in prepupae compared to larvae and notably pupae reflects that the infection takes place in the larval stages but primarily is revealed in prepupae accumulating on tree trunks as they attach firmly to the substrate. In comparison, the larval stages may fall to the ground when moribund or dead. In some cases, fungus infection is carried over from the larval stage to the pupal stage. E.g., of the 31 prepupae infected by fungus in the case above, 5 specimens died after successfully developing into pupae.

We isolated *Lecanicillium lecanii* and *L. muscarium* from *H. axyridis*, but several isolates remain unidentified as they do not fit into current descriptions of *Lecanicillium* species based on micromorphology (Zare & Gams, 2001). In the unidentified isolates of *Lecanicillium* from *H. axyridis*, the secondary branching of phialides is similar to *L. lecanii*, whereas the conidia size and phialide dimensions are closer to *L. muscarium*. Whether these isolates are new species awaits the results of molecular analysis.

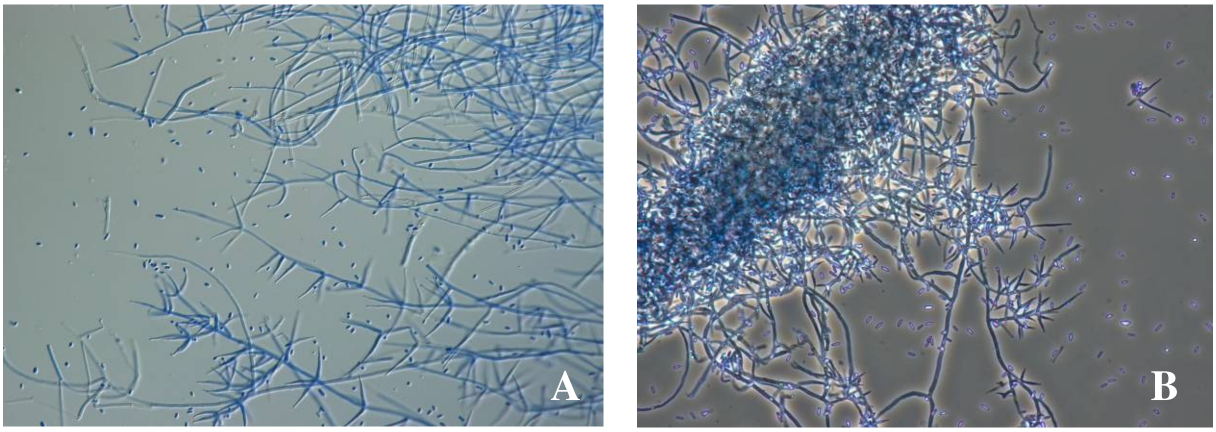

Figure 2. The micromorphology of *L. muscarium* (A) and *L. lecanii* (B) isolated from *H. axyridis*. Photos: Richard A. Humber.

In addition to the mitosporic fungal species described above, the parasitic fungus *Hesperomyces virescens* (Laboulbeniales) was recorded for the first time in Europe in overwintering adults collected in spring 2009 from a house in Hohenstein near Frankfurt, Germany. One infected specimen of *H. axyridis* was found among 113 individuals collected. We have not yet found this fungal species in Danish *H. axyridis*, although some of our samples of overwintering *H. axyridis* also contained *A. bipunctata* heavily infected by *H. virescens*.

Figure 3. Thalli (arrow) of *Hesperomyces virescens* on the posterior part of the elytra of a female *H. axyridis*.
Photo: Tove Steenberg

In conclusion, a variety of entomopathogenic fungi were found to infect field populations of *H. axyridis* soon after the arrival of the new coccinellid species in Denmark. The natural occurrence appears widespread, and larvae (including prepupae), pupae and adults are infected. In some cases, high infection levels are reached. At present we do not know whether this group of natural enemies is able to regulate the population density of *H. axyridis*.

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The harlequin ladybird (*Harmonia axyridis*) in Denmark: spread, phenology, colour forms and natural enemies in the early phase of establishment

Tove Steenberg*[1] & Susanne Harding[2]
[1]*Department of Integrated Pest Management, University of Aarhus, Skovbrynet 14, DK-2800 Kgs. Lyngby, Denmark;* [2]*Department of Agriculture and Ecology, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark*
**Author for correspondence: Tove.Steenberg@agrsci.dk*

Abstract: *Harmonia axyridis* arrived in Denmark in 2006, was established by 2007 and now has spread to the southern and eastern part of the country. The rate of spread in Denmark has been surprisingly low, and except for a few strongholds with large populations *H. axyridis* has mainly been recorded as single specimens. Studies of the phenology of *H. axyiridis* in Denmark indicate bivoltinism and show that it is currently not well adapted to Danish conditions. The frequency of occurrence of four colour forms has not changed within the first two years after its establishment in Denmark. At present, f. *succinea* is the dominant colour form (~94%) and the nominate colour form f. *axyridis* is rare (0.6% prevalence). Among the native natural enemies interacting with *H. axyridis* are phorid flies (*Phalacrotophora* sp.), entomopathogenic fungi and the hymenopteran parasitoid *Dinocampus coccinellae*.

Key words: adaptation, spread, phenology, colour form, polymorphism, natural enemies, *Phalacrotophora*, *Dinocampus coccinellae*

Introduction

The harlequin ladybird, *Harmonia axyridis* is an invasive species that has recently arrived in Denmark. A few adult specimens were recorded in 2006, and by October 2007 large numbers of larvae, pupae and adults were found in parks and other outdoor locations in Copenhagen. In addition, the first aggregations of overwintering adults were reported from indoor locations (Steenberg & Harding, 2008). We subsequently initiated a project with the overall aim to study how *H. axyridis* adapts to the Danish environment.

Geographical spread (including the rate of spread), phenology, colour form polymorphism and interactions with native natural enemies are useful characters when evaluating the adaptation of a polymorphic species to new conditions and are included in the study. This paper provides an overview of results as concerns the initial phase of the invasion in Denmark.

Material and methods

Spread

Mapping of the geographical spread of *H. axyridis* in Denmark is based on verified records of the species submitted by the public to the Danish harlequin ladybird website launched in May 2008 (www.dpil.dk), records submitted to two other websites (www.fugleognatur.dk and www.obsnatur.dk) and personal observations from sampling in different parts of the country. The electronic recording scheme on the Danish harlequin website provides data on location, numbers found, life stages, colour forms and habitats.

Phenology

Observations on the phenology of *H. axyridis* were made in central Copenhagen, where a number of locations in parks and other green areas were visited at short intervals from early March to the end of October. At each inspection, observations were made of the presence of eggs, larvae, pupae and adults. Habitats ranged from wooded areas to reed beds and ruderal areas. In 2007 and 2009 *H. axyridis* was active also in November and December, so the observation season was prolonged accordingly. Further details of this part of the study can be found in Steenberg & Harding (2009a).

Colour forms

Harmonia axyridis is a polymorphic species occurring in several distinct colour forms. If different colour forms adapt differently to an environment, changes over time in the relative frequencies of colour forms can be expected during the initial phase of establishment. We recorded the relative frequencies of four colour forms of *H. axyridis* (f. *succinea*, f. *spectabilis*, f. *conspicua* and f. *axyridis*) from a number of sites in Copenhagen from late autumn 2007 and onwards (Steenberg & Harding, 2008). To avoid bias, sampling focused primarily on pupae from different plants and from a large area at the different sampling sites. Samples were taken in spring as well as in autumn. In addition, adults were sampled primarily from overwintering aggregations.

Natural enemies

Natural enemies of native coccinellids have the potential to adapt to new species such as *H. axyridis*. We surveyed samples of larvae, pupae and adults for infection by pathogenic microorganisms and for evidence of parasites and parasitoids utilizing *H. axyridis* as a host. The work on entomopathogenic fungi has been reported elsewhere (Steenberg & Harding, 2009b; Steenberg & Harding (ibid.)). In late June 2009, species of *Phalacrotophora* (Diptera: Phoridae) were found to parasitize pupae of *H. axyridis* (Disney & Beuk, 1997). Pupae of *Coccinella septempunctata*, *Adalia bipunctata* and *H. axyridis* occurring simultaneously at two sampling sites in Copenhagen in July and August 2009 were subsequently collected to compare parasitation levels in the three coccinellid species. Pupae were incubated separately at room temperature and numbers of fly puparia emerging were recorded.

Results and discussion

Spread

Most Danish records of *H. axyridis* to date are adults found as single specimens. From the first record of the species in two light traps in the summer of 2006, *H. axyridis* has now been recorded from southern and eastern Denmark with a major hotspot in greater Copenhagen (per December 2009). Two further records from 2009 outside the distribution area (locations in central and north Jutland) with certainty represent spread via imported commodities – from South Europe and USA, respectively. The main source of the invasion of Denmark however is thought to be adult *H. axyridis* flying in from further south in Europe (Steenberg & Harding, 2009a). A map of the current geographical spread of *H. axyridis* in Denmark is continuously updated at the Danish harlequin ladybird website (www.dpil.dk). The course of spread in 2006 to 2009 is illustrated in Steenberg et al. (2009).

There is no doubt that the species is spreading in Denmark. However, the rate of spread has been lower than expected compared to reports from other European regions such as the United Kingdom (Brown et al., 2008). *Harmonia axyridis* was recorded in Hamburg – approx. 150km south of Denmark – already in 2002 where it was present in large numbers (Tolasch, 2002). However, it took five years before it was recorded 40km south of the border in Schleswig-Holstein in 2007 (A. Loomans, pers. comm.), and in accordance, overwintering adults were not found north of the border until early 2008. We also expected the species to spread rapidly to South Sweden from the Copenhagen area, where it has now been present in high numbers for a couple of years. Although *H. axyridis* was recorded from an apartment in Malmø, Sweden, on two occasions in 2007 (T. Hägg, pers. comm.) we have not yet recorded it outdoors on subsequent visits to this city which is situated merely 25km to the east of Copenhagen.

The slow spread that has been observed since the species arrived in Denmark is not likely due to lack of food as abundant aphid populations were available in spring, summer and/or autumn in the period 2007 to 2009. Future monitoring of the spread of *H. axyridis* will allow us to better evaluate whether the invasion rate really is slowing down.

Phenology

Observations in 2007, 2008 and 2009 showed that *H. axyridis* appears to be bivoltine in Denmark unlike most native species of coccinellids (Steenberg & Harding, 2009). Overwintering adults become active in April and – depending on spring temperatures and food availability – the first new generation emerges as adults in mid June and onwards. Adults of the second generation will emerge from early August onwards. The study of the phenology in summer is made difficult by overlapping of generations, particularly concerning the emergence of the second generation. Depending on the temperature in late autumn and early winter, adults will initiate overwintering from late October and onwards.

It is clear from observations in the first three autumns following the species establishment in Denmark that it is not yet adapted to Danish conditions. In 2009, eggs were being laid as late as 19 September, and in 2007 active larvae were found and pupation took place in November-December. In both years last-instar larvae and pupae failed to develop into overwintering adults and died in late autumn and early winter. Also, a fraction of those adults emerging late in season probably failed in finding sufficient food to survive the winter period.

Colour forms

The mean percentages of the four different colour forms have not changed significantly since the first data published in Steenberg & Harding (2008). Of more than 4500 pupae sampled in a number of sites in Copenhagen in June-July 2009 94.4% was f. *succinea*, 3.9% were f. *spectabilis*, 1.1% were f. *conspicua* and 0.6% were f. *axyridis* (Fig.1). However, for f. *axyridis* there are marked differences between locations. Sampling on a number of occasions from May 2008 to October 2009 in a single location showed that 1.5-2.5% of *H. axyridis* were f. *axyridis*.

The current European records of f. *axyridis* to our knowledge include records almost exclusively from border areas of the European invasion. It has been reported from Germany, Denmark, Czech Republic, The Netherlands, Poland and Bulgaria (Tolasch, 2002; Steenberg & Harding 2008; Nedved et al., 2008, C. L. Raak-van den Berg & A. J. M. Loomans, pers. comm., P. Ceryngier, pers. comm., Tomov et al., 2009). Populations from these border areas also frequently show very high proportions of the red colour form f. *succinea* (Steenberg & Harding 2008; Burgio et al., 2008; Tomov et al. 2009).

Although the two melanic colour forms f. *spectabilis* and f. *conspicua* are genetically dominant to f. *axyridis* (Tan, 1946), at present there are no signs of f. *axyridis* disappearing from the Danish population as a result of selection. We are currently studying life traits of this relatively rare colour form in laboratory experiments.

Offspring from our laboratory crossings of f. *spectabilis* and f. *axyridis* have included specimens of f. *spectabilis* where the diameter of the anterior set of spots is similar to that of the posterior set of spots and thus much smaller than usually seen in this colour form (Fig. 2). Interestingly, we also recorded a similar specimen from the location where the prevalence of f. *axyridis* is 1.5-2.5%. Tan (1946) illustrated a similar specimen from crossings of f. *spectabilis* and f. *axyridis* as an example of mosaic dominance. The finding of such specimens in the field implies that the local prevalence of f. *axyridis* is of a magnitude to produce examples of mosaic dominance in the population.

Figure 1. *Harmonia axyridis*, forma *axyridis*. Photo: Susanne Harding.

Figure 2. Forma *spectabilis* sampled from a location with a f. *axyridis* prevalence of 1.5-2.5%. The specimen to the left appears to display mosaic dominance. Photo: Tove Steenberg.

Natural enemies

A range of species of entomopathogenic fungi have been isolated from larvae, pupae and adults of *H. axyridis* (Steenberg & Harding, 2009; Steenberg & Harding, ibid.). A few specimens of the hymenopteran parasitoid *Dinocampus coccinellae* have been reared from adult *H. axyridis*.

Pupae of all three species of coccinellids were parasitised by phorid flies of the genus *Phalacrotophora*. The overall prevalence was 2.7% in *H. axyridis* (N=4508), 55.4% in *C. septempunctata* (N=233) and 11.4% in *A. bipunctata* (N=404). In one location, where only *H. axyridis* pupae could be found, the prevalence of *Phalacrotophora* was 23% (N=52), but throughout the sampling season the prevalence was mostly well below 5%. *Phalacrotophora* sp. appears to adapt to the new host also in terms of number of fly puparia developing per pupa (*C. septempunctata*: mean 11.5, range 1-29; *A. bipunctata*: mean 2.8, range 1-6, *H. axyridis*: mean 4.6, range 1-24) and in % fly emergence from puparia (*C. septempunctata*: 68.3%, *A. bipunctata*: 43.3%, *H. axyridis*: 48.8%). Future sampling will show whether the prevalence of native natural enemies will increase as *H. axyridis* becomes more abundant and whether this will cause problems for native coccinellids.

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Is *Harmonia axyridis* really eating *Adalia bipunctata* in the wild?

Alison Thomas[1], Styliana Philippou[1], Remy Ware[2], Heather Kitson[1] & Peter Brown[1]
[1]Dept Life Sciences, Anglia Ruskin University, UK; [2]Dept Genetics, University of Cambridge, UK
Corresponding author: Alison.Thomas@anglia.ac.uk

Abstract: Preliminary work was conducted to identify a PCR based method for detection of *Adalia bipunctata* in the predatory ladybird *Harmonia axyridis*. A primer pair (Ab35) was identified which amplified well a microsatellite marker in the genome of *A. bipunctata* but not that of *H. axyridis*. Controlled laboratory studies were conducted when *H. axyridis* fourth instar larvae were fed on *A. bipunctata* eggs or first instar larvae. A single first instar larva or seven to ten eggs could be detected two hours post-feeding, suggesting that if a field collected *H. axyridis* larva had consumed an *A. bipunctata* larva or a number of eggs within two hours before collection, this intraguild predation could be detected. Of 112 field collected *H. axyridis* larvae tested, one revealed the presence of *A. bipunctata.*

Key words: *A. bipunctata*, *H. axyridis*, PCR, predation, digestion

Introduction

Harmonia axyridis is a predatory coccinellid, native to central and eastern Asia. It has been available since 1916 in the US and more recently in Europe (e.g. France, Belgium and Holland) for use as a biological control agent of pest aphids and scale insects. In all these countries *H. axyridis* has established. In Great Britain it was inadvertently introduced in 2004 (Majerus & Roy, 2005) and has since spread across much of the country (Brown *et al.*, 2008). Although primarily aphidophagous, *H. axyridis* feeds on a wide array of foods and may be threatening a variety of insects, including butterflies, lacewings and ladybirds.

Since the arrival of *H. axyridis* in the UK, the national ladybird survey (www.ladybird-survey.org) has recorded a fall in observations of the two spot ladybird, *Adalia bipunctata*. Three years of surveying seven sites close to Cambridge, UK, where *H. axyridis* is common, has revealed a decline in *A. bipunctata* numbers by 72% (2008 numbers compared to 2006) (Peter Brown, *unpublished data*). Intraguild competition is hypothesized to be a major reason for this observed decline, either as the result of competition for shared resources or direct predation of *A. bipunctata* by *H. axyridis*. This paper describes the development of a DNA based method that can detect predation of *A. bipunctata* by *H. axyridis*.

The Polymerase Chain Reaction (PCR) is a powerful tool for detecting very low levels of DNA. The rationale for this detection method was to extract DNA from field collected *H. axyridis*. PCR reactions would be performed using a pair of PCR primers that are specific for the *A. bipunctata* genome. Thus successful PCR amplification would only occur if *A. bipunctata* was present in the gut of *H. axyridis*. There is a precedent for using PCR to investigate the diet of a variety of insects, reviewed by King *et al.* (2008). A set of PCR primers which amplify microsatellite loci in the *A. bipunctata* genome, was developed by Hadrill *et al.* (2002). Initially our aim was:

- to identify a pair of primers that were specific to the *A. bipunctata* genome
- to determine the sensitivity of *A. bipunctata* specific primers in terms of (i) how much *A. bipunctata* has to be eaten to be detected and (ii) for how long after eating can *A. bipunctata* be detected
- to test the method on field collected *H. axyridis*

Material and methods

Samples

Field collected *H. axyridis* larvae were gathered from lime trees in four churchyard sites in eastern England (Worlington, St. Ives, Wilburton and Fordham) in late June/early July 2008 and 2009 and in early October 2009. Laboratory reared *H. axyridis* and *A. bipunctata* were bred in the Department of Genetics, University of Cambridge. Ladybird samples were frozen at -80°C until DNA extraction.

Feeding experiments

A single first instar *A. bipunctata* larva or 1, 5, or 10 *A. bipunctata* eggs were fed to a single fourth instar *H. axyridis* larva, which had been starved for 24hrs prior to the experiment. Following consumption of the larva or eggs *H. axyridis* larvae were frozen at -80°C, immediately or after 2, 4, 8, or 24hrs. All feeding experiments were done in replicates of five.

DNA extraction

The larvae were snap frozen in liquid nitrogen. 208µl of the extraction buffer (containing 0.01g of CHELEX-100, 7µl of IM DTT and 1µl of 25mg/ml proteinase K) was then added to each larva in a separate 1.5ml eppendorf tube. Each larva was crushed with a plastic pestle, the tube vortexed for 10 seconds and then incubated at 56°C for 1.5hrs. After vortexing for 10 seconds each sample was incubated at 96°C for 10 minutes and then centrifuged at 13000rpm for 5 minutes. The supernatant (containing the extracted DNA) was poured into a fresh eppendorf tube and the samples refrigerated at 4°C.

PCR amplification

Each PCR reaction (25µl) contained (10.7µl) double distilled water, 10x amplification buffer (2µl) (supplied by the manufacturer with the enzyme), 25mM of $MgCl_2$ (2.5µl), Q solution (4µl), 100µM of each dNTP (0.2µl), 10µM of each primer, AB35F (GAAATTGTTAGGTTAGGATCGGAAG) & AB35R (ATTCACTGTGCGGAGTATTACGTTC) (2µl), 0.1U Taq DNA Polymerase (5u/µl) and 2µl of extracted DNA. Amplification was performed in a TECHNE TC 412 thermocycler with the following cycling conditions: an initial denaturation at 94°C for 3mins, followed by 35 cycles of denaturation at 94°C for a minute, annealing at 60°C for a minute and DNA extension at 72°C for a minute. There was a final extension step at 72°C for 10 minutes, then the products were stored at 4°C. The PCR products were separated with agarose gel electrophoresis. The PCR products were mixed with 3µl Gel Loading dye and 6µl loaded on a 2.5% agarose gel which contained 1µl Ethidium Bromide (10mg/ml). The gels were run at 70% volts for 2.5 hours in 1% TBE buffer. The PCR products were then photographed on a UV transilluminator for PCR analysis.

Results

Specificity of* A. bipunctata *Ab35 PCR primers

When these primers were tested with DNA extracted from whole *A. bipunctata* and *H. axyridis*, successful amplification of a polymorphic electrophoretic band of approximately 215-255bp was only achieved with *A. bipunctata* DNA (Figure 1). *Adalia decempunctata* is also commonly found in the same habitats as *A. bipunctata*. No amplification was detected when the AB35 primers were also tested with DNA extracted from whole *A. decempunctata*. This result showed that PCR primers AB35 are specific to *A. bipunctata*.

Sensitivity of PCR detection

PCR results are summarized in Table 1. When one first instar *A. bipunctata* larva was presented to a starved fourth instar *H. axyridis* larva, *A. bipunctata* DNA was successfully detected for a 2 hour period (Figure 1). All five replicates yielded a strong electrophoretic band when the *H. axyridis* larvae were frozen immediately. Four of the five replicates yielded weaker bands when the larvae were frozen after two hours. No successful amplification was obtained using samples frozen after 4, 8 or 24 hours.

No detection was made when a single *A. bipunctata* egg was fed to starved *H. axyridis* fourth instar larvae. When five *A. bipunctata* eggs were fed to *H. axyridis* larvae a PCR product was detected when the larvae were frozen immediately that consumption had finished. There was also successful PCR amplification when seven to ten eggs were fed to *H. axyridis* larvae and the larvae frozen immediately. In the '2hr' feeding experiment, successful amplification occurred in one instance, when eight *A. bipunctata* eggs were fed to *H. axyridis* larvae.

Table 1: Detection of *A. bipunctata* DNA in the gut of *H. axyridis* fourth instar larvae 0, 2, 4, 8, 24hrs after consumption. Each feeding trial was done five times.

A. bipunctata	**0hrs**	**2hrs**	**4hrs**	**8hrs**	**24hrs**
First instar larvae	5/5	4/5	-	-	-
5 eggs	4/5	-	-	-	-
10 eggs*	5/5	1/5	-	-	-

*The five replicates variously consumed 7-10 of the 10 eggs offered

Detection of A. bipunctata in field collected H. axyridis

112 field collected *H. axyridis* larvae were tested. PCR amplification of *A. bipunctata* was detected for one *H. axyridis* larva collected from St Ives churchyard in summer 2008.

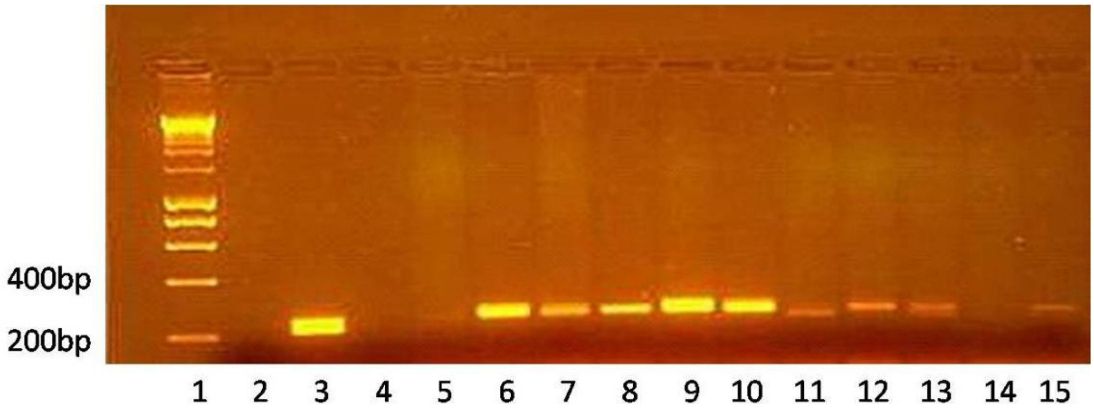

Figure 1. PCR amplification of DNA from first instar *A. bipunctata* larvae fed to fourth instar *H. axyridis* larvae. Lanes 6-10 show the presence of *A. bipunctata* DNA in the *H. axyridis* guts 0 hours and lanes 11-15 2 hours after feeding. Lane 3 shows the positive PCR control, amplification of known *A. bipunctata* DNA. Lane 4 shows the reaction blank when no DNA was included in the PCR reaction. Lane 5 shows the negative PCR control, when only *H. axyridis* DNA was included in the PCR reaction.

Discussion

This paper presents preliminary work to identify a rapid and efficient PCR based method for detection of *A. bipunctata* in the predatory ladybird *H. axyridis*. Hadrill *et al.* (2002) developed PCR primers for amplification of a set of microsatellites in the genome of *A. bipunctata*. Primer pair Ab35 amplified well a $(GA)_n$ micosatellite in *A. bipunctata* but not in the genome of *H. axyridis* or *A. decempunctata*, a common congener in the eastern England habitats sampled. Controlled laboratory studies when *H. axyridis* fourth instar larvae were fed on *A. bipunctata* eggs or first instar larvae produced promising results. A single first instar larva or seven to ten eggs could be detected two hours post-feeding, suggesting that if a field collected *H. axyridis* larva had consumed an *A. bipunctata* larva or a number of eggs within two hours before collection, this intraguild predation could be detected. Of 112 field collected *H. axyridis* larvae tested, one revealed the presence of *A. bipunctata*.

This work has demonstrated that a PCR based analysis of the gut contents of *H. axyridis* has the power to reveal intraguild predation of *A. bipunctata* under field conditions. It is possible that a detection rate of 0.89% suggested by this preliminary work is an under-estimation, as many factors will influence the detection period for prey in a predator's gut. For example, the amplicon size of the prey PCR marker will affect detectability. Shorter fragments have been shown to be detected for longer periods after ingestion (Hoogendoorn & Heimpel, 2002). The size of the polymorphic PCR marker used in this reported work was 215-255bp. Other insect predation work has targeted 100 -150bp fragments of both the internal transcribed spacer of ribosomal gene complex (ITS-1) and mitochondrial cytochrome oxidase 1 gene (CO1). Both these PCR targets are present in multiple copies in the genome; another factor which could increase the detection period. The Ab35 primers used in this reported work target a single copy genomic site. Ambient temperature, because of its effect on digestive enzymes, would be expected to influence detection time of prey, as would the age of egg masses and satiation state of *H. axyridis*.

In summary, this study suggests that a PCR based method could provide a realistic and reliable measure of IGP once certain constraints relating to experimental design are addressed. Consideration should be given to targeting a shorter DNA fragment in the *A. bipunctata* mitochondrial genome. Zindel *et al.* (2010) have designed PCR primers that amplify a 100bp fragment of cytochrome oxidase 1 of *A. bipunctata*. Alternatively a different molecular approach could be used. Hautier e*t al.* (2008) have recently shown the effectiveness of gas chromatography-mass spectrometry (GC-MS) to detect the alkaloid adaline from *A. bipunctata* in the gut of fourth instar *H. axyridis* larvae. The detection period was long, up to 96 hours after ingestion of a single egg or first instar *A. bipunctata* larva. However GC-MS cannot distinguish between adaline produced by *A. bipunctata* and *A. decempunctata*. In the context of this study it is desirable to have a separate molecular marker for *A. bipunctata* and *A. decempunctata* as they are found in the same habitats.

Consideration should also be given to using real time PCR (RT-PCR), instead of standard PCR, to amplify the prey target DNA. RT-PCR is more sensitive, and so could extend the detection period of *A. bipunctata* in the gut of *H. axyridis*. DNA produced by RT-PCR can be quantified, so quantitative estimation of prey biomass consumed could also be deduced. RT-PCR can also detect and quantify multiple sequences in a single reaction; thus has the potential to be simultaneously informative about IGP of both *A. bipunctata* and *A. decempunctata* by *H. axyridis*.

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Investigating global invasion routes of the harlequin ladybird (*Harmonia axyridis*) using mtDNA

Cathleen E. Thomas[1], Eric Lombaert[2], Remy Ware[3], Arnaud Estoup[4] & Lori Lawson Handley[1]
[1]Department of Biological Sciences, University of Hull, Hull, HU6 7RX, UK; [2]IBSV, INRA, 06903 Sophia-Antipolis, France; [3]Department of Genetics, University of Cambridge, Cambridge, CB2 3EH, UK; [4]CBGP, INRA, 34988 Montferrier-sur-Lez, France
Corresponding author: c.e.thomas@2007.hull.ac.uk

Abstract: Although species invasions have important ecological and economical consequences, there is still much we do not understand about why only certain species become successful invaders, and what impacts they have on other species. Molecular techniques can be used to gain vital information on invasion dynamics, so as part of an ongoing study, we have used mitochondrial DNA sequence data to investigate the global invasion of *Harmonia axyridis*, and particularly the characteristics of the founding population(s). This will also complement data currently being gathered on microsatellite genotype and endosymbiont presence to provide a powerful dataset for understanding the invasion history of this species, and, more broadly, to attempt to determine what factors might make an invasive species successful.

Key words: *Harmonia axyridis*, invasive species, mitochondrial DNA

Introduction

Species invasions are one of the greatest threats to biodiversity, agriculture and human health. Two major goals of invasion biology are (1) to identify sources and routes of spread of an invasive species and enable better controls to be implemented, and (2) to understand why certain species become invasive and what impacts they have on the native species with which they interact.

Molecular genetic data can be used to infer origins and routes of spread (e.g. Guillemaud *et al.,* 2010), estimate the number of founders (e.g. Ficetola *et al.,* 2008), and test whether multiple introductions from different geographic regions has contributed to invasion success via increased diversity and/or novel genotype combinations (e.g. Kolbe *et al.,* 2004; Dlugosch & Parker, 2008). The successful invasion of *Harmonia axyridis* (Coleoptera: Coccinellidae) on different continents provides a unique opportunity to investigate the invasion history of a highly invasive species, and to attempt to identify common factors underlying invasion success.

The specific aims of our ongoing study are (1) to investigate the invasion history of *H. axyridis* by identifying potential source population(s) and invasion routes using maternally inherited mitochondrial DNA (mtDNA), and (2) to determine whether invasions are characterised by high mtDNA haplotype diversity in the founding population, which could be linked to multiple introductions of diverse founding lineages. We hypothesise that the founding populations of *H. axyridis* in the invasive range will have high genetic diversity in comparison to native populations, and that invasive populations will be founded from multiple sources.

Material and methods

A 586bp region of the mtDNA *COI* gene was amplified using PCR and sequenced in 111 individuals representing 9 native populations (from Russia, China, Japan and Kazakhstan), 8 invasive populations (from Belgium, Brazil, UK, South Africa, USA) and 3 biocontrol populations (from France and Belgium). Sequences were aligned and summary statistics such as haplotype diversity were calculated using MEGA 4 (Tamura et *al.,* 2007). A haplotype network was constructed using the programme Network 4.5.1.0 (Bandelt *et al.,* 1999). Another 240 individuals are currently being sequenced and the complete dataset will be analysed using an Approximate Bayesian Computation based method implemented in DIY ABC (Cornuet *et al.,* 2008).

Results and discussion

Preliminary results show 24 unique haplotypes, with one common haplotype (H1) shared between 17 of the 20 populations studied. This includes populations from all native and invasive countries analysed (except one population in Russia, one in Brazil and one in USA, though this could be due to the small sample size tested in these populations thus far), and all biocontrol populations. The remaining haplotypes were related to H1 by a maximum of two mutational steps. This low level of divergence between individuals from different regions is characteristic of a recent range expansion.

Little geographical structure has been detected thus far as many individuals share haplotype H1, however, rare haplotypes have been found that are shared between two population pairs: Washington (USA) and Helderberg (South Africa); and Colorado (USA) and Curitiba (Brazil). These data suggest at least two maternal founders within the USA, and that the USA may be a source for invasive populations in South Africa and Brazil. Further studies are currently ongoing using microsatellite markers to confirm this result.

Preliminary results of the current study also indicate haplotype diversity is quite high in invasive populations. For example, four haplotypes were found in nine individuals from South Africa, and three haplotypes were found in eight individuals from the UK – both populations which have been founded within the last five years. Further work will be done to determine whether high diversity is related to multiple introductions within invasive populations.

When interpreting results generated from mtDNA markers in insects it is also important to consider the presence of endosymbiont bacteria. As both mtDNA and endosymbiont bacteria are inherited in the same way (through the cytoplasm of the maternal egg cell) there is some linkage between the distribution of haplotypes and presence of bacteria that does not relate to geographical distribution (e.g Johnstone & Hurst, 1996; Engelstädter & Hurst, 2007). As *Wolbachia* sp. and other endosymbionts have been detected in native, biocontrol and invasive populations of *H. axyridis* (Aebi and Zindel, 2010), it is vital to take this into account when interpreting invasion history from mtDNA. By combining data from complementary studies on mtDNA, microsatellite loci and endosymbiont presence we can obtain a powerful insight into the invasion dynamics of this highly invasive species.

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Occurrence of the harlequin ladybird *Harmonia axyridis* (Pallas, 1773) (Coleoptera: Coccinellidae) in Bulgaria

Rumen Tomov[1], Katya Trencheva[1], Georgi Trenchev[1] & Marc Kenis[2]
[1]University of forestry, 10, Kliment Ohridski blvd, 1756 Sofia, Bulgaria; [2]CABI Europe-Switzerland, 1, rue des Grillons, 2800 Delémont, Switzerland
Corresponding author: rtomov@yahoo.com

Abstract: Surveys were carried out in Bulgaria in 2009 to monitor the presence of the invasive harlequin ladybird, *Harmonia axyridis* (Pallas, 1773) (Coleoptera: Coccinellidae). Ladybirds were collected by beating the branches of trees and shrubs and sweeping grasslands throughout the country. The species was found in 17 localities in Bulgaria: Ardino, Belogradchik, Bladoevgrad, Botevgrad, Dupnitsa, Elin Pelin, Gabrovo, Kresna defile, Montana, Pravets, Smolyan, Sofia, Varna, Veliko Turnovo, Velingrad, Vidin and Vratsa,. Except for the natural location Kresna defile, *H. axyridis* occurred mainly in urbanised landscapes. The species was more often found in Western part of the country. The species was found exclusively on broadleaved trees heavily infested by aphids: *Acer pseudoplatanus* L., *Cornus sanguinea* L., *Fraxinus excelsior* L., *Paliurus spina-christi* Mill., *Populus nigra* L., *Quercus rubra* L., *Quercus cerris* L., *Tilia cordata* Mill. and *Tilia tomentosa* Moench. The pathway of introduction of the ladybird in Bulgaria is not clearly known. Although releases of *H. axyridis* were carried out in Bulgaria and Greece in the 1990s, the present invasion is most probably due to populations coming from the West. The invasion of *H. axyridis* in Bulgaria is still at an early stage and despite the first observation of the species in 2008, it seems that it started to spread in summer 2009 from populations of Sofia.The infestation by *Eucallipterus tiliae* (L.) on *Tilia cordata* Mill. clearly facilitated the natural spread of *H. axyridis* in Bulgaria.

Key words: Coccinellidae, *Harmonia axyridis*, exotic species, biological invasions, establishment, Bulgaria

Introduction

The multicolored Asian lady beetle, or harlequin ladybird, *Harmonia axyridis* (Pallas, 1773) (Coleoptera: Coccinellidae), is native to central and eastern Asia (Dobzhansky, 1933). The species has been widely used as biological control agent against aphids and coccids in various crops in North America (Koch, 2003) and Europe (Brown *et al.,* 2008). Recently, feral populations of *H. axyridis* have been found in many European countries (Brown et al., 2008). *Harmonia axyridis* has spread in Europe at a very fast rate occupying different habitats and it is now regarded as established in more than 15 countries (Brown *et al.,* 2008; Tomov *et al.,* 2009).

Due to its predatory and competitive abilities the species may have strong negative effects on biodiversity (Majerus *et al.,* 2006; Koch and Galvan, 2007). In a risk assessment of 31 exotic natural enemies of pest species used in biological control in Europe, *H. axyridis* had the second highest environmental risk index (van Lenteren *et al.* 2003, van Lenteren *et al.* 2008). In North America it is considered as a human nuisance (Huelsman et al. 2002) and has been reported to damage fruit crops, especially grapes (Pickering *et al.,* 2006). No biological control is as yet available and farmers in US spray chemicals against *H. axyridis*. Several management strategies against *H. axyridis* are being developed and have been reviewed by Kenis *et al.* (2008).

In Bulgaria, *H. axyridis* was first recorded in 2008 when two adults were found in Sofia (Tomov *et al.,* 2009). In the framework of the project “Alien terrestrial arthropods and their impact on biodiversity in Bulgaria (ATARTIB), a long term study on the ecological impact of harlequin ladybird *H. axyridis* was initiated. In the present paper we report the first data on the distribution of *H. axyridis* in Bulgaria.

Material and methods

Surveys to monitor the distribution of *H. axyridis* in Bulgaria were carried out from late April to late November, 2009. Forty five permanent plots were established near Sofia at the foot of the Vitosha mountain and surveyed monthly for the occurrence of *H. axyridis* and native ladybirds. The plots were situated in semi-natural habitats and consisted of 15 broadleaved hedges (about 50 meters), 15 conifer sites (8-10 large trees per site) and 15 grassland sites.

Additional surveys were conducted in environments and on plant species potentially suitable for *H. axyridis* (20 urban sites of Sofia and 45 localities outside Sofia situated in different climatic zones of Bulgaria) (Figure 1). Each locality was sampled twice in a season during the periods July-August and September-October. Five –six plants per plant species were chosen at random in the sites.

Ladybirds were collected by beating the branches of trees and shrubs with a stick above a white 80 x 100cm beating tray. Grassland habitats were sampled by sweeping 60 times with a net of 35cm diameter.

Results

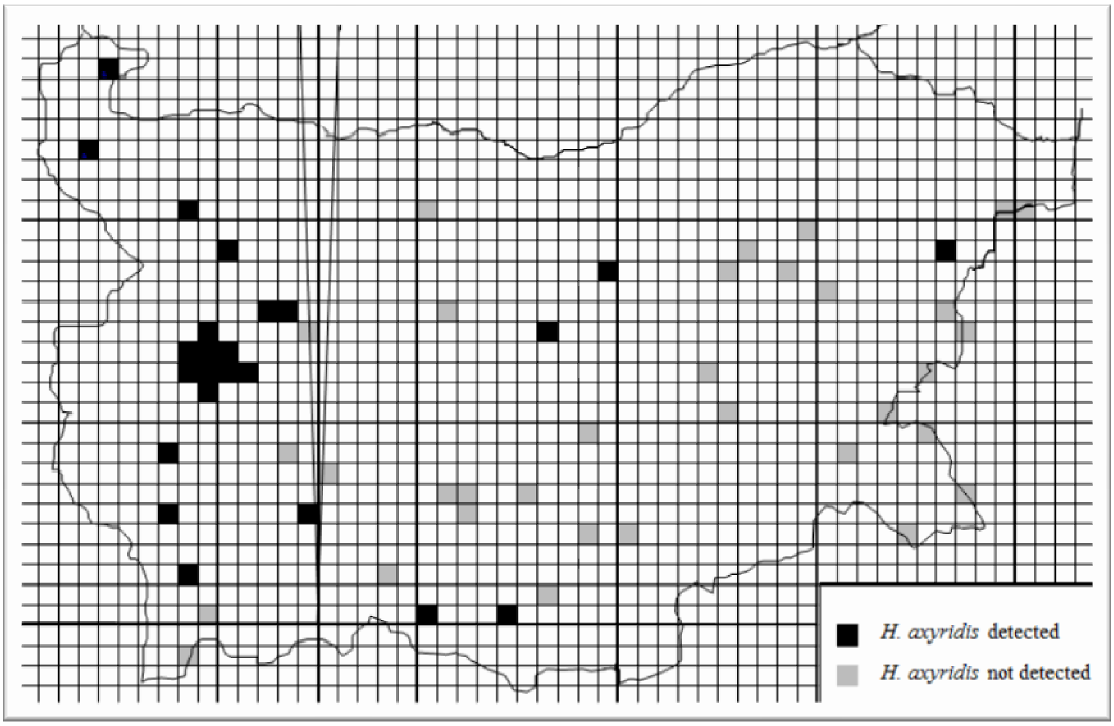

Figure 1. Observed distribution of *H. axyridis* in Bulgaria 2009 (10 x 10km^2 UTM grid)

Large populations of *H. axyridis* were found in all urban areas of Sofia in 2009. A gradual expansion of the species was observed during the period July – November 2009. Until mid August, *H. axyridis* was frequent in the south parts of Sofia (Mladost, Studentski grad and Hipodruma) while, in the north parts of Sofia (Kremikovtsi, Buchovo), the beetle was found only later in the year. Surveys made at the end of August failed to locate the ladybird at locations at 5-10 km distance from Sofia - Bistritsa, Zheleznitsa and Kostinbrod, but during our survey in October the species was found in all villages near Sofia.

By the end of November 2009, surveys made in urban and natural environments outside Sofia resulted in positive observations in 16 of the 45 surveyed localities (Table 1). The species was more often found in Western part of the country. A map of the known distribution of *H. axyridis* in Bulgaria as of the end of 2009 is presented in fig. 1.

During our surveys in 2009, *H. axyridis* was not detected in the following localities: Pleven, Troyan, Etropole, Omurtag, Turgoviste, Veliki Preslav, Shumen, Smyadovo, Staro Oryahovo in North Bulgaria; Kavarna, Balchik, Obzor, Nesebar, Burgas, Sozopol, Tsarevo at the Black Sea coast; Sandanski, Petrich, Belovo, Kostenetz, Parvomay, Plovdiv, Sadovo, Stara Zagora, Sliven, Yambol, Asenovgrad, Devin, Kurdzhali, Haskovo, Harmanli, Malko Tarnovo, Sredets in South Bulgaria.

Table 1. Host plants, prey and localities in Bulgaria where *H. axyridis* was recorded in 2009

Plant	Prey	Location/Date of collection/ Sample size (L-larvae, P-pupae, A – adults)
Tilia cordata Mill.	*Eucallipterus tiliae* (L.)	Sofia July – November (more than 650 individuals); Dupnitsa, 11.08.09 (3 L, 2 A); Blagoevgrad , 11.08.09 (2 L, 1 A); Botevgrad , 22.08.09 (5 L, 3 P, 5 A); Smolyan, 29.08.09 (4 L, 5 P, 2 A); Gabrovo, 23.09.09 (6 P, 7 A); Elin Pelin, 25.09.09 (10 L, 2 P, 7 A); Velingrad, 12.10.09 (1 P, 6 A); Montana, 15.10.09 (7 L, 17 A); Vratsa, 15.10.09 (6 L, 2 P, 7 A); Vidin 16.10.09 (1 L, 7 P, 9 A); Belogradchik, 16.10.09 (9 L, 14 A)
Tilia tomentosa Moench.	*Eucallipterus tiliae* (L.)	Ardino, 30.08.09 (3 L)
Acer pseudoplatanus L.	*Drepanosyphum* sp.	Sofia, 31.08.09 (19 L, 9 P, 24 A)
Quercus rubra L.	*Myzocallis walshii* (Monell)	Sofia, 31.08.09 (5 L, 8 P, 32 A); Varna, 26.07.09 (2 A)
Quercus cerris L.	*Tuberculatus querceus* Kalt.	Veliko Turnovo, 22.09.09 (7 L, 2 P, 9 A)
Populus nigra L.	*Chaitophorus versicolor* Koch.	Sofia, 16.10.09 (17 A)
Fraxinus excelsior L.	*Prociphilus fraxinifolii* Riley	Sofia, 20.10.09 (7 L, 26 A)
Paliurus spina-christi Mill.	*Aphis* sp.	Kresna defile, 17.07.09 (7 L), 20.09.09 (2 A)
Cornus sanguinea L.	*Anoecia corni* Fabricius	Sofia, 05.11.09 (2 L, 2 P, 17 A)
Aesculus hipocastanum L.	no aphid prey detected	Sofia, 16.08.09 (1 P)
Salix fragilis L.	no aphid prey detected	Sofia, 30.08.09 (4 A)

With the exception of Kresna defile, all localities where *H. axyridis* was recorded were urban areas. Semi-natural areas surveyed regularly during the season failed to provide *H. axyridis.* The invasive beetle was found mainly on trees heavily infested by aphids. The host plant, aphid prey species and number of specimens collected are reported in table 1. A total of 230 larvae, 119 pupae and 666 adults were collected in the study. Two adults on *Salix fragilis* L. and one pupae of *Aesculus hipocastanum* L. were found without aphid infestation but the trees were situated close to heavy infested *Tilia cordata*. Reproducing populations of the ladybird were detected at all sites except in Varna.

Discussion

Harmonia axyridis was not found during our surveys in spring 2009 but this may be due to the fact that, at that time, efforts were focused mainly on natural environments while later surveys showed that, for the moment, *H. axyridis* occurs mainly in urban areas in Bulgaria.

The pathway of introduction of the species in Bulgaria is not known. Flightless populations of *H. axyridis* were released for biological control purposes in small fields in the regions of Sofia and Pazardzik in 1993 (Radeva pers. comm). Successful overwintering of introduced adults was observed in the field and these succeeded in suppressing aphid populations during the period 1993-1994. However, there is no published data concerning the presence of *H. axyridis* in Bulgaria after this period. The species is not included in the list of ladybirds in the Eastern Rhodopes (Jordanova, 2004) and during our survey the species was not detected in the region of Pazardzik. Thus, it is very unlikely that populations observed since 2008 originate from individuals introduced 15 years earlier.

Harmonia axyridis was also introduced in Greece in the 1990s (Katsoyannos *et al.,* 1997). However, two facts suggest that the natural spreading from Greece to Bulgaria in unlikely: (1) There is little evidence of establishment of the species in Greece (Brown et al., 2008); (2) During our survey made in August, the species was not detected in cities in the Southwest Bulgaria, close to Greece (Sandanski, Pertich and Melnik). It was found in Smolyan and Ardino (cities in Rodopa Mountain) but was not found in other locations nearby (Kardzaly, Devin). In Smolyan, ladybirds were collected near a flower shop selling imported ornamental plants, which suggests that the introduction in this area could have been made by human activities.

It is most likely that *H. axyridis* invaded Bulgaria from the West, for example from Serbia, where it was reported in 2008 (Thalji and Stojanovic, 2008). Examples from Western and Central Europe showed that the spread of *H. axyridis* can be very rapid. The beetle can spread both by human activities and by natural spreading. We suppose that the presence of the species in Varna, Smolyan and Kresna defile is due to human transport since these locations are touristic places. In the Sofia region, the beetle was found nearly exclusively in cities and villages and not on trees planted near roads between the cities or in natural environments, suggesting also transportation by human activities. Since *H. axyridis* was first found in Sofia in 2008 and seemed to spread from the city centre during 2009, it is possible that the ladybird was accidentally introduced in the city a couple of years ago or may be even only in 2008.

The heavy infestation of *Tilia cordata* by the aphid *Eucallipterus tiliae* probably facilitated the natural spreading of *H. axyridis* observed in Bulgaria in 2009, despite the fact that several other aphid species on broadleaved trees were found as prey for the beetle (Table 1). In summer 2009, we observed the expansion of *E. tiliae* from urban to suburban areas in the region of Sofia, in parallel to that of the ladybird. *Tilia cordata* is distributed mainly in the

Western part of Bulgaria but also at the North Black Sea coast and the Strandzha Mountain (South-East Bulgaria) and we may thus expect to find the beetle in all these regions in the near future.

Despite a first observation of *H. axyridis* in 2008, we believe that the invasion of the species in Bulgaria is still in a very early stage, for two reasons. Firstly, surveys were made in 45 localities outside Sofia but *H. axyridis* was detected in only 16 of them. Many trees infested by aphids had no *H. axyridis*, in particular in Stara planina Mountain (Pravets, Etropole, Troyan), Strandzha Mountain (Malko Turnovo, Gramatikovo, Sredets) and Black sea coast (Obsor, Burgas, Sozopol, Tsarevo). Secondly, even in Sofia, the distribution of the beetle is still very patchy, and several *Tilia cordata* trees heavily infested by aphids were found without *H. axyridis* in the suburban areas, at 200 m distance from trees infested by the ladybird.

The number of generations in Bulgaria was not studied. Larvae, pupae and adults were recorded from mid July to the end of November. Considering that four overlapping generations of *H. axyridis* have been reported in Greece (Kastoyannos et al., 1997), we may expect at least three generations in Bulgaria.

Bearing in mind the variety of habitats and plants occupied by *H. axyridis* in Europe (Adriaens *et al.*, 2008, Brown *et al.,* 2008, Burgio *et al.,* 2008) many aphid species can be considered as suitable prey for *H. axyridis* in Bulgaria. Applications for the commercial release of the species were refused by the Bulgarian Regulatory Authority in 2009 but, considering the rate of spread observed in Europe in recent years (Brown *et al.,* 2008) and the rapid increase in abundance in Bulgaria in 2009, we may expect that the beetle will invade the whole country in a few years.

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Suitability of diverse prey species for development of *Harmonia axyridis* and the effect of container size

Dita Ungerová[1], Plamen Kalushkov[2], Oldřich Nedvěd[1,3*]
[1]University of South Bohemia, Faculty of Science, Branišovská 31, 37005 České Budějovice, Czech Republic; [2]Institute of Zoology, Bulgarian Academy of Sciences, Blvd. Tzar Osvoboditel 1, Sofia, Bulgaria; [3]Biology Center, Institute of Entomology, Branišovská 31, 37005 České Budějovice, Czech Republic
**Corresponding author: nedved@prf.jcu.cz*

Abstract: Larval development time and fresh body mass of newly emerged adults are widely used quantitative parameters characterizing food suitability. These parameters were measured in the ladybird *Harmonia axyridis* with nine different aphid species. We calculated suitability parameter SL = *m*/*t* (mass divided by developmental time), and ranked the aphid species studied accordingly: *Aphis philadelphi* (2.6), *Aphis fabae* on *Rumex* (2.5), *Aphis spiraephaga* (2.0), *Acyrthosiphon pisum* (1.8), *Rhopalosiphum padi* (1.7), *Acyrthosiphon ignotum* (1.6), *Dysaphis plantaginea* (1.5), *Hyadaphis tataricae* (1.4), *Aphis sambuci* 2009 (1.3), *Aphis sambuci* 2008 (1.1). Conspecific eggs were moderately suitable (1.7). When larvae were reared together in 0.5l glass jars, the developmental parameters were better than when reared individually in 7 or 15cm Petri dishes in combination with most aphid species. Sexual differences in fresh mass (females being 1.1 to 1.2 times heavier) but not in developmental time were found. Some prey species which are well suitable for large larvae were found not so good for young ladybird larvae due to the large body size of these aphids.

Key words: developmental time, body mass, aphids, Coccinellidae, essential prey

Introduction

Food suitability for larval development may be characterized by several parameters such as larval development time, survival rate and fresh mass of pupa or newly emerged adult (Kalushkov, 1998, Kalushkov & Hodek, 2004, 2005, Ueno, 2003).

Harmonia axyridis is a very polyphagous ladybird (Alhmedi *et al.*, 2008; Berkvens *et al.*, 2008, van Lenteren *et al.*, 2008), similar to the native European species *Coccinella septempunctata*, *Propylea quatuordecimpunctata* and *Adalia bipunctata*. While the performance of *A. bipunctata* strongly differs when fed on different aphid species (Fig. 1; Kalushkov, 1998), the results for *C. septempunctata* across aphid species are much more consistent (Kalushkov & Hodek, 2004). Both larval developmental time and adult mass varied very little in *P. quatuordecimpunctata* fed with six aphid species (Fig. 1; Kalushkov & Hodek, 2005). There was larger variability in adult mass than in developmental time in all species studied, at least when an extremely unsuitable prey was excluded. Measured as the range divided by the mean, it was 8% in developmental time in *C. septempunctata* and *P. quatuordecimpunctata* and 22% in *A. bipunctata*, while it was 11, 20 and 53% in adult mass in respective three species. *Acyrthosiphon ignotum* was the best prey for *Adalia bipunctata* (Kalushkov, 1998) followed by *Aphis fabae*, while *Aphis spiraephaga* was unsuitable prey for *A. bipunctata* when fed exclusively. *Harmonia axyridis* reared on *Acyrthosiphon pisum* showed a shorter developmental time and a heavier pupal mass than their siblings on *Aphis craccivora* and on artificial diet (Ueno, 2003).

The study of the effect of prey density (*Myzus persicae*) on duration of larval development and teneral adult live mass of *H. axyridis* showed a concave decreasing function between the two measures and sexual difference in the mass achieved (Hukusima & Ohwaki, 1972).

Development duration of *H. axyridis* fed with *Myzus persicae* was 14-16 days and with *Amphorophora oleraceae* 15 days at 25°C. At 30°C, development duration was 8 and 7 days when larvae were fed with these two aphids, respectively, 8 days with *Acyrthosiphon pisum*, *Macrosiphum rosae*, *Megoura viciae*, 10 days with *Periphyllus californensis*, 12 days with *Aphis pomi*, and 13 days with *Brevicoryne brassicae* and *Hyalopterus arundinis* as two least suitable prey (Hukushima & Kamei, 1970). Other aphid species classified as essential food (sensu Hodek, 1996) for *H. axyridis* were *Aphis craccivora* (Mogi, 1969), *Capitophorus eleagni* (Osawa, 1992), *Nasonovia lactucae* (Hukushima & Ohwaki, 1972), *Rhopalosiphum prunifoliae* (Okamoto, 1961) and *Schizaphis graminum* (Fye, 1981). *Harmonia axyridis* preyed upon *Hyalopterus pruni*, *Stegophylla guercicola*, *Aphis nerii*, and *Aphis asclepiadis* in the field (Hesler & Kieckhefer, 2008). More arboreal hosts (trees and shrubs) and associated preys utilized by *H. axyridis* were listed by Koch *et al.* (2006).

The main aim of this study was a comparison of suitability of several prey species for larval development of the introduced ladybird species *H. axyridis*, measured as the developmental time and adult mass. We also performed a comparison between males and females, and we found unexpected differences between rearing containers of different size: small and large Petri dishes and a large jar. We included some prey considered suboptimal for certain ladybird species and therefore expected large differences in the developmental parameters of *H. axyridis*.

Material and methods

Adults of *Harmonia axyridis* (form *succinea*) were collected in South Bohemia in the Czech Republic and reared at 25°C and 18L:6D hour photoperiod on *Acyrthosiphon pisum*. Their progeny were reared under the same conditions, but fed with one of the following aphid species on their host plants: *Aphis philadelphi* on *Philadelphus coronarius*, *Aphis fabae* on *Rumex acetosa*, *Aphis spiraephaga* and *Acyrthosiphon ignotum* on *Spiraea vanhoutei*, *Acyrthosiphon pisum* on *Vicia faba*, *Rhopalosiphum padi* on *Padus racemosa*, *Dysaphis plantaginea* on *Malus sylvestris*, *Hyadaphis tataricae* on *Lonicera tatarica*, and *Aphis sambuci* on *Sambucus nigra*, and also with conspecific eggs completed with a wet piece of cotton. All aphids were wild caught, except *Acyrthosiphon pisum* on *Vicia faba* which was a laboratory culture. Aphids were provided ad libitum.

We observed the larvae once daily, and recorded their developmental time (t, in days) from hatching of the 1st instar to pupation. Fresh mass of newly emerged adults (m, in mg) was measured with electrobalances (precision 0.1mg). The sex of emerged adults was determined according to head colouration (McCornack *et al.*, 2007). We invented a parameter representing a suitability level SL = m/t (mass divided by developmental time), and ordered the aphid species accordingly.

H. axyridis larvae were reared in three parallel treatments = container sizes: a) in a small Petri dish (diameter 7cm), b) in a large Petri dish (15cm), and c) in a glass jar (0.5l) covered by a nylon mesh. Various containers were used when trying to save space in incubators and to find an optimal rearing design. Ladybird and aphid densities per space unit were somewhat smaller in larger containers. Results were analysed using analysis of variance; all data given is presented as means ± standard error.

Results

Minimum (11.4mg) and maximum (39.4mg) adult fresh mass among all 699 individuals measured differed strongly, as well as the group (Table 1) means (17.1 to 33.0mg). Variation in the developmental time of the larval stage was smaller, with absolute minimum 9 days and maximum of 21 days, while treatment means lay within a narrow range from 9.5 to 13.8 days, except for the larvae fed with toxic *Aphis sambuci* (16.3 days).

Sex and prey effects on adult mass

A two way ANOVA analysing sex and prey species effects on adult mass was highly significant (F_1=47, $p<10^{-6}$) between males (21.9±0.3mg) and females (25.2±0.3mg) and across the prey species (F_9=33, $p<10^{-6}$), with no interaction (F_9=1, p=0.34). However, we did not manage to make all combinations of prey species and container size in the experimental design (see Table 1), so that we analysed mass measured in 7cm Petri dishes, separately, as this container was used in combination with all prey species. There was a highly significant difference (F_1=36, $p<10^{-6}$) between males (22.6±0.4mg) and females (26.1±0.5mg) and significant heterogeneity among the prey species (F_9=49, $p<10^{-6}$), with no interaction (F_9=1, p=0.80). Means ± SE for individual prey species in 7cm Petri dishes, sexes pooled, were: *A. philadelphi*: 31.5±0.6mg; *A. fabae*: 27.5±0.5mg; *A. spiraephaga*: 25.4-0.5mg; *A. pisum*: 22.3±0.6mg; *R. padi*: 20.8±0.8mg; *H. axyridis* eggs: 19.9±0.9mg; *D. plantaginea*: 19.8±0.9mg; *A. sambuci*: 19.3±0.8mg; *A. ignotum*: 19.1±0.9mg; *H. tataricae*: 16.9±0.7mg.

Sex and prey effects on developmental time

A two way ANOVA analysing sex and prey species effects on developmental time was more weakly significant (F_1=6, p=0.01) between males (12.1±0.1 days) and females (12.4±0.1 days) and strongly significant across prey species (F_9=28, $p<10^{-6}$), with no interaction (F_9=2, p=0.10). Because we did not manage to make all combinations of prey species and container size in the experimental design (see Table 1), we analysed developmental time measured in 7cm Petri dishes separately. There was a weakly significant difference (F_1=5, p=0.04) between males (12.4±0.1 days) and females (12.5±0.1 days) and highly significant heterogeneity among the prey treatments (F_9=38, $p<10^{-6}$), with no significant interaction effects (F_9=2, p=0.07). Means ± SE for individual prey species in 7cm Petri dishes, sexes pooled, were: *A. fabae*: 11.0±0.1 days; *H. axyridis* eggs: 11.9±0.4 days; *A. ignotum*: 12.0±0.4 days; *R. padi*: 12.1±0.3 days; *H. tataricae*: 12.2±0.3 days; *A. philadelphi*: 12.3±0.1 days; *A. spiraephaga*: 12.6±0.2 days; *A. pisum*: 12.7±0.2 days; *D. plantaginea*: 12.9±0.3 days; *A. sambuci*: 16.2±0.3 days.

There was a weak correlation between the developmental time and adult mass. When calculated between males reared in 7cm Petri dishes, the equation was $\text{time}=11.3+3693\cdot\text{mass}^{-2.70}$; R^2=0.19. For females, it was $\text{time} = 10.6+192\cdot\text{mass}^{-1.44}$; R^2=0.21. There was a similar weak correlation between the two parameters when calculated using mean values for each prey species (Figure 1).

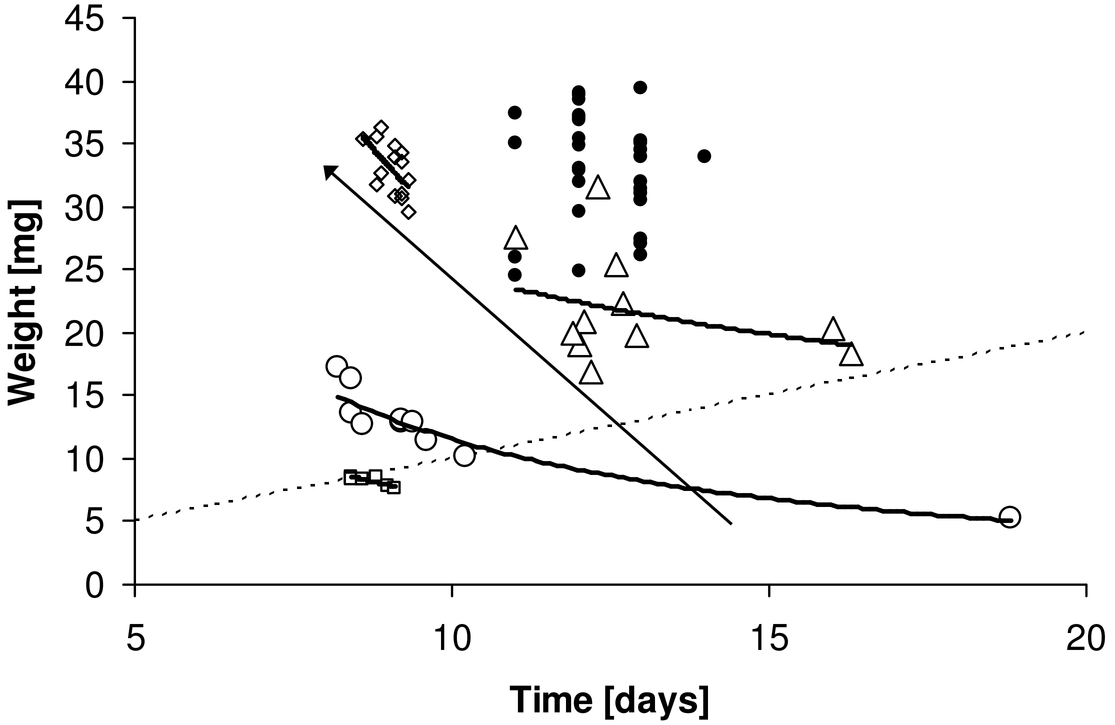

Figure 1: Relationship between larval developmental time and adult fresh mass in three aphidophagous ladybirds reared each on several different aphid species. *Harmonia axyridis*: triangles (present study, 7cm Petri dishes, sexes pooled); *Coccinella septempunctata*: diamonds (Kalushkov and Hodek 2004); *Adalia bipunctata*: circles (Kalushkov 1998); *Propylea quatuordecimpunctata*: squares (Kalushkov and Hodek 2005). Non-linear relationships were estimated by power functions. Dotted line: isoline representing suitability parameter SL=1; arrow: direction of linear increase of suitability parameter. Should the extremely weakly suitable prey species be excluded, the relationships would be roughly linear, parallel to the arrow. Dots represent individual measurements of *H. axyridis* females reared on *Aphis philadelphi* (the mean together with males is the upper triangle).

Sex and container size effects on adult mass

A two way ANOVA analysing sex and container size effects on adult mass was highly significant ($F_1=62$, $p<10^{-6}$) between males and females and among the container types ($F_2=21$, $p<10^{-6}$), with no interaction ($F_2=0.1$, $p=0.88$). In small (7cm) Petri dishes, the mean mass ±SE was 24.4±0.3mg; in 15cm Petri dishes 21.2±0.4mg; in 0.5l jars 23.9±0.4mg. However, not all combinations of prey species and container size were used in the experimental design (see Table 1), so that we also analysed only those four species which were tested in all three container sizes (*A. ignotum*, *A. sambuci*, *D. plantaginea*, and *H. tataricae*). There was a highly significant difference ($F_1=42$, $p<10^{-6}$) between males and females and among the container types ($F_2=57$, $p<10^{-6}$), with no interaction ($F_2=0.03$, $p=0.97$). In small (7cm) Petri dishes, the mean mass ±SE was 18.8±0.4mg; in 15cm Petri dishes 21.2±0.4mg; in 0.5l jars 25.1±0.4mg.

Table 1: Developmental time of larvae, fresh mass of adults, and suitability parameter of *Harmonia axyridis* fed with diverse prey species and reared at three different container sizes (Petri dish, diameter 7cm; Petri dish, diameter 15cm; glass jar, 500ml). Numbers of individuals per treatment are given in parentheses in the last column but are the same for developmental time, Fresh adult mass and Suitability. Means ± standard deviations.

Prey	Sex	Developmental time (days)			Fresh adult mass (mg)			Suitability (mg/day) and (N)		
		PD7	PD15	jar500	PD7	PD15	jar500	PD7	PD15	jar500
A. philadelphi	M	12.3±0.6	-	-	29.5±4.2	-	-	2.4 (24)	-	-
	F	12.3±0.8	-	-	33.0±4.4	-	-	2.7 (30)	-	-
A. fabae	M	10.9±0.8	-	-	25.9±3.4	-	-	2.4 (36)	-	-
	F	11.1±0.9	-	-	29.4±4.1	-	-	2.6 (31)	-	-
A. spiraephaga	M	12.9±1.4	-	-	23.2±3.4	-	-	1.8 (27)	-	-
	F	12.4±1.1	-	-	27.1±3.3	-	-	2.2 (34)	-	-
A. pisum	M	12.7±1.5	-	-	21.4±3.5	-	-	1.7 (33)	-	-
	F	12.8±1.4	-	-	23.4±5.3	-	-	1.8 (29)	-	-
R. padi	M	12.0±1.2	-	12.4±1.6	18.6±2.0	-	18.2±3.2	1.6 (7)	-	1.5 (22)
	F	12.2±0.8	-	12.4±1.7	23.3±1.8	-	21.7±3.6	1.9 (6)	-	1.8 (22)
A. ignotum	M	12.0±1.5	10.9±1.3	9.5±0.8	17.3±3.1	18.4±3.4	26.0±4.7	1.4 (8)	1.7 (42)	2.7 (18)
	F	12.0±1.9	10.8±1.3	9.5±0.6	20.5±3.5	22.0±4.1	32.6±2.1	1.7 (10)	2.0 (49)	3.4 (15)
D. plantaginea	M	12.5±0.5	12.8±1.2	11.5±0.8	19.5±3.0	22.2±3.9	25.2±3.5	1.6 (6)	1.7 (10)	2.2 (13)
	F	13.3±1.5	13.3±1.3	12.1±0.8	20.1±3.2	23.0±5.2	24.6±5.3	1.5 (6)	1.7 (10)	2.0 (8)
H. tataricae	M	11.9±1.0	11.2±1.3	12.4±1.2	15.1±1.4	21.7±2.8	19.4±2.5	1.3 (8)	1.9 (13)	1.6 (23)
	F	12.4±1.2	11.8±1.8	13.0±1.5	18.5±3.3	27.1±5.0	22.4±2.9	1.5 (9)	2.3 (12)	1.7 (23)
A. sambuci	M	16.3±2.2	12.9±1.2	12.1±2.4	17.1±2.2	19.6±3.1	24.3±4.9	1.0 (13)	1.5 (10)	2.0 (22)
	F	16.0±1.4	13.4±1.7	12.0±1.5	21.5±4.5	20.2±3.2	27.7±5.5	1.3 (13)	1.5 (10)	2.3 (30)
H. axyridis eggs	M	11.4±1.4	-	-	19.5±4.1	-	-	1.7 (13)	-	-
	F	13.8±1.5	-	-	21.1±2.7	-	-	1.5 (4)	-	-

Sex and container size effects on developmental time

A two way ANOVA analysing sex and container size effects on developmental time detected no significant difference ($F_1=2$, $p=0.18$) between males and females, but a highly significant difference among the container types ($F_2=18$, $p<10^{-6}$), with no interaction ($F_2=0.1$, $p=0.91$). In small (7cm) Petri dishes, the mean time ±SE was 12.5±0.1 days; in 15cm Petri dishes 11.5±0.1 days; in 0.5l jars 11.8±0.1 days. Because not all combinations of prey species and container size were used in the experimental design we also analysed those four species which were used in all three container sizes. There was no difference ($F_1=1$, $p=0.41$) between males and females and a highly significant difference among the container types ($F_2=35$, $p<10^{-6}$), with no interaction ($F_2=0.2$, $p=0.79$). In small (7cm) Petri dishes, the mean time ±SE was 13.7±0.3 days; in 15cm Petri dishes 11.5±0.1 days; in 0.5l jars 11.7±0.1 days.

Discussion

As is typical for coccinellids, females were consistently heavier than males (see Honěk, 1996), but the sexual difference was almost undetectable in developmental time. Females were 1.12 times heavier than males fed with *Aphis philadelphi*, 1.17x on *Aphis spiraephaga*, 1.13x on *Aphis fabae*, 1.14x on *Aphis sambuci*, 1.15x on *Hyadaphis tataricae*, 1.20x on *Acyrthosiphon ignotum*, 1.09x on *Acyrthosiphon pisum* and1.19x on *Rhopalosiphum padi*. In a previous study on *H. axyridis* (Hukusima & Ohwaki, 1972), females were in average 1.27x heavier than males. The difference was higher at higher aphid density. The following differences were found in other aphidophagous ladybirds: 1.20x and 1.18x in *Propylea japonica* (Kawauchi, 1978, 1979), 1.25x in *Adalia bipunctata* (Mills, 1981), 1.09x in *Hippodamia quinquesignata* (Kaddou, 1960).

Large differences existed in fresh adult mass (17–32mg, 65% of mean value) in ladybirds fed by different aphid species, and smaller in the developmental time (11–13 days, 16% of mean value, when excluding 16 days on less suitable *A. sambuci*). The two parameters were negatively correlated, but only slightly and non-linearly (Fig.1; triangles). Thus both parameters should be measured in comparative studies, and our suitability parameter SL = m/t may unify them to a single measure. The suitability parameter has a unit miligrams per day. It means that it represents analogy to growth rate or average daily mass gain.

There was a similar relationship between the two parameters measured by Kalushkov (1998) in *A. bipunctata*: higher variability in adult mass than developmental time, negative relationship between them (closer than observed in *H. axyridis*) and non-linear relationship when a weakly suitable prey was included (Fig. 1; open circles). In *Propylea quatuordecimpunctata*, only six aphid species were tested, resulting in a narrow range of both developmental time and adult mass (Kalushkov & Hodek, 2005) again negatively correlated (Fig. 1; squares). The variability was rather low in *Coccinella septempunctata* despite higher number of prey species studied (Fig. 1; diamonds). It seems that *H. axyridis* has highest phenotypic plasticity among these four aphidophagous ladybirds (cf. Lombaert *et al.*, 2008). There was no or slightly negative relationship between mass and developmental time within treatments and sexes (Fig. 1; dots).

Aphis sambuci exhibited a very low suitability for *H. axyridis* according to both parameters measured, with *H. axyridis* fed this prey exhibiting prolonged development and low adult mass), and also in low consumption rate and low survival rate (about 50%). *A. sambuci* is toxic to the ladybird *Coccinella septempunctata* (Hodek, 1956, Nedvěd & Salvucci, 2008) but more suitable for *Adalia bipunctata* (Nedvěd, 1999). Earlier in the season *A. sambuci* is not so unsuitable for *H. axyridis*. Probably host plants do not have such a high concentration of the defensive compounds sambucine and sambunigrine in May (2009) compared to June or July (2008; compare cf. Hodek, 1956). *C. septempunctata* was surprisingly unable to distinguish *A. sambuci* as a toxic prey in laboratory choice experiments when it was offered without the host plant (Nedvěd & Salvucci, 2008). The second least suitable prey was *Hyadaphis tataricae* which is covered and protected by rich waxy exudations. Conspecific eggs were moderately suitable for *H. axyridis* larvae.

Among the prey species tested in the present study, three were previously tested (Kalushkov, 1998) as food for ladybird *A. bipunctata*, which overlaps widely in habitat usage with introduced *H. axyridis* (Kenis *et al*., 2009) and is reported to decline after *H. axyridis* spreaded (Brown *et al*., 2009). *A. ignotum* was suitable prey for both ladybirds and thus they may compete for it, while *A. fabae* from *Atriplex sagittata* and *A. spiraephaga* were unsuitable prey species for *A. bipunctata* according to Kalushkov (1998). However, Nedvěd (1999) found larvae of *A. bipunctata* developing on *A. fabae* on several plant species, suggesting that there might also be competition with *H. axyridis* for this prey on some host plants.

There were four common aphid species tested both in the present study and in the study of Kalushkov & Hodek (2004) with *C. septempunctata*: suitable *A. ignotum*, *A. spiraephaga*, *A. pisum* and less suitable *A. fabae*. Sharing several common suitable prey species and avoiding the same prey species suggest possibility of strong competition for food between these two polyphagous ladybird species.

When larvae were reared together in 0.5l glass jars, both developmental parameters were generally better than when reared together in 15cm diameter Petri dishes and those were better than when larvae were reared individually in 7cm Petri dishes (SL values): on *Aphis sambuci* 2.2; 1.5; 1.3; *Hyadaphis tataricae* 1.6; 2.1; 1.4; *Dysaphis plantaginea* 2.1; 1.7; 1.5; and the best improvement was in *Acyrthosiphon ignotum* 3.1; 1.9; 1.6. This improvement might be associated with some level of cannibalism in the case of weakly suitable *A. sambuci* because the conspecifics represent a better prey, but this explanation is not viable for ladybirds reared on other aphid species.

Two effects of the space available (volume or surface) might be distinguished: space for movement and population density, sensed as the number of encounters with other larvae or detection of their trace pheromone. Ladybird larvae probably do not depend on large space for movement if prey is abundant. Satiated larvae spent most time motionless. Evans (1991) demonstrated that the presence of a conspecific or a heterospecific larva decreases the development rate and the mass increase of third and fourth instar larvae of *Hippodamia convergens* was reduced by 30% when reared in pairs compared to solitary larvae. The effect of ladybird population density in our case will be tested in the future through measuring the space and the surface of the three different containers and separating ladybird larvae in precise numbers. Effect of prey density must be also carefully controlled, so that laboratory stock of aphids will be used.

However, we suggest another, more simple explanation of different SL values in different containers – the effect of air humidity. While the jars were covered by a nylon mesh providing good ventilation and thus suitable humidity, Petri dishes filled with leaves of host plants retained very high humidity. Although ladybird larvae and aphids provided as food need water supply, too much air humidity may be deleterious. This hypothesis will be also tested in the future.

We observed that some prey species which are well suitable for large ladybird larvae are not so good quality for young larvae due to the large body size of these aphids and hence difficult catching and killing.

We consider the ranking of diverse aphid species according to their suitability to *H. axyridis* comparable to the ranking according to their suitability to other ladybird species (*A. bipunctata* in Kalushkov, 1998; *C. septempunctata* in Kalushkov & Hodek, 2004; *P. quatuordecimpunctata* in Kalushkov & Hodek, 2005) in slightly different laboratory conditions, and thus we believe that different ladybirds utilize the same prey species differently – aphids highly suitable for one ladybird (e.g. *A. spiraephaga* for *H. axyridis*) may be of low quality for another (*A. spiraephaga* for *A. bipunctata*). Good rating in one parameter does not mean good result in the other but the two we used (developmental time and mass) were correlated. We want to emphasize that rearing conditions other than prey species, such as type of container, also appear to be important in determining larval fitness.

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Adaptation of native parasitoids to a novel host: the invasive coccinellid *Harmonia axyridis*

Remy Ware[1], Laura-Jane Michie[1], Tomoki Otani[2], Emma Rhule[1] & Richard Hall[3]

[1]*Department of Genetics, University of Cambridge, Downing Street, Cambridge, CB2 3EH, UK;* [2]*Peterhouse, Cambridge, CB2 1RD, UK;* [3]*Odum School of Ecology, 140 E. Green Street, University of Georgia, Athens, GA 30602-2202, USA*
Corresponding author: rlw29@cam.ac.uk

Abstract: In its introduced range, the invasive coccinellid *Harmonia axyridis* (Coleoptera: Coccinellidae) threatens many non-pest insects through competition and predation, and this raises the need for appropriate control measures to be investigated. One strategy could be to consider the introduction of natural enemies (predators, parasites and pathogens) that regulate *H. axyridis* populations in its native range. Indeed, escape from natural enemies is likely to have contributed to its invasive success (the so-called 'enemy release hypothesis'). However, re-uniting *H. axyridis* with its own enemies requires rigorous and time-consuming risk assessment to ensure there are no unwanted side effects on native species. Moreover, the introduction of alien enemies may be unnecessary if the organisms that attack native ladybirds in Britain also attack harlequins. Here we present field data which indicates that two species of parasitoid wasp: *Dinocampus coccinellae* (Hymenoptera: Braconidae) and *Oomyzus scaposus* (Hymenoptera: Chalcidoidea); and two species of parasitoid fly: *Phalacrotophora fasciata* and *Phalacrotophora berolinensis* (Diptera: Phoridae) may be adapting to *H. axyridis* in Britain as a novel and abundant host. This may provide some level of natural population control.

Key words: Coccinellidae, biological control, *Dinocampus coccinellae,* enemy release hypothesis, *Harmonia axyridis*, *Oomyzus scaposus*, parasitoids, *Phalacrotophora berolinensis*, *Phalacrotophora fasciata*

Introduction

The invasive coccinellid *Harmonia axyridis* is native to central and south-east Asia, but has had a long history of use in the biological control of aphids and coccids (Koch, 2003). Following repeated introductions into North America as early as 1916, it was reported as being established in the wild in the late 1980s (Chapin & Brou, 1991). The species was commercially available in mainland Europe since 1982, and is now established in over thirteen European countries (Brown *et al.*, 2008a). In 2004, it reached Great Britain, most likely via multiple routes (Majerus *et al.*, 2006a), and has since spread and increased in number rapidly (Brown *et al.*, 2008b), prompting fears that many native insects may become threatened through competition and predation (Ware *et al.*, 2005).

Many factors are thought to account for the invasiveness of *H. axyridis* outside its native range, including a polyphagous diet, its ability to thrive in a variety of habitats, high reproductive potential, broad climatic tolerance, wide dispersal capabilities, and its propensity to act as intraguild predator to other members of the aphidophagous guild (Majerus *et al.*, 2006b; Ware *et al.*, 2005; Roy & Wajnberg, 2008, Babendreier *et al.*, 2010). A further explanation may lie in the 'natural enemy release hypothesis' (Torchin *et al.*, 2003), which states that invasive species are often more successful than conspecifics from the native range because they escape the natural enemies (predators, parasites and pathogens) which would ordinarily attack them. Furthermore, such agents occupying the introduced range will not be co-evolved with the invasive species, and will likely take time to adapt to a novel prey/ host.

In its native range, *H. axyridis* is known to be attacked by several parasitoids, including: *Dinocampus coccinellae* (Hymenoptera: Braconidae) (Liu, 1950; Maeta, 1969; Kuznetsov, 1997); *Homalotylus flaminius* (Hymenoptera: Chalcidoidea) (Kuznetsov, 1997); *Oomyzus scaposus* (*=Tetrastichus coccinellae*) (Hymenoptera: Chalcidoidea) (Kuznetsov, 1997); *Phalacrotophora philaxyridis* (Diptera: Phoridae) (Disney, 1997) and *Medina (=Degeeria) luctuosa* (Diptera: Tachinidae) (Kuznetsov, 1997). It is also infected by a male-killing *Spiroplasma* at varying prevalence (Majerus *et al.*, 1999), and ocassionally by the entomopathogenic fungus *Beauveria bassiana* (Kuznetsov, 1997).

A broad suite of natural enemies is reported attacking coccinellids in Europe, including hymenopteran and dipteran parasitoids; parasitic mites and worms; and fungal and bacterial pathogens (Majerus, 1994; Ceryngier & Hodek, 1996; Klausnitzer & Klausnitzer, 1997). The braconid wasp *D. coccinellae* has been recorded from adults of at least 18 species of coccinellid in Europe, all from the subfamily Coccinellinae (Ceryngier & Hodek, 1996), and coccinellid larvae and pupae are parasitised by several species of small chalcid wasps of the genera *Homalotylus* and *Tetrastichus* (Majerus, 1994; Ceryngier & Hodek, 1996). European *Phalacrotophora* attacking coccinellid pupae include *P. fasciata, P. berolinensis, P. beuki, P. delageae, P. paradoxa, P. pictofasciata* and *P. spectabilis* (Disney & Beuk, 1997), with only the former two species occurring in Great Britain (Disney et al., 1994). Tachinid flies of the genus *Medina* spp. (*Medina luctuosa* and/or *M. seperata*) have been found parasitising adults of 12 species of coccinellid in Europe. European coccinellids are also host to ecto-parasitic mites of the genus *Coccipolipus* (Acarina: Podapolipidae), with the best researched being the widely distributed *C. hippodamiae*, known to cause female sterility in *Adalia bipunctata* (Hurst et al., 1995; Webberley et al., 2004). Parasitic nematodes from the Allantonematidae (e.g. *Parasitilenchus coccinellinae*) and the Mermithidae (e.g. *Mermis* spp.) are known as natural enemies of coccinellids in the wild (Iperti, 1964; Ceryngier & Hodek, 1996). Fungal pathogens are widely regarded as important natural enemies of coccinellids, with the most significant groups being the Hypocreales (e.g. *Beauveria bassiana*) and the Laboulbeniales (e.g. *Hesperomyces viriscens*) (Roy & Cottrell, 2008). Most studies of bacterial infections of Coccinellidae concern the male-killing bacteria, and, since the first discovery in *Adalia bipunctata*, male-killers of four bacterial groups (*Wolbachia*, *Rickettsia*, *Spiroplasma* and flavobacteria) have been identified from eight other species of coccinellid in Europe (Majerus, 2006; Weinert *et al.*, 2007).

It seems likely that a number of the natural enemies that ordinarily attack coccinellids in Europe will begin to utilise *H. axyridis* as a novel and abundant host, particularly holarctic species known to attack *H. axyridis* in Asia, and those species with a somewhat cosmopolitan host range. Since the arrival of *H. axyridis* in Great Britain in 2004 (Brown *et al.*, 2008b), we have been in a unique position to monitor the ecology of an invasive species from its point of arrival, including its interactions with natural enemies. Not only does this provide an excellent opportunity to study evolution in action, but such interactions may, in the future, become significant in terms of population control. In this paper we report the first instance of the parasitoids *P. fasciata*, *P. berolinensis*, *D. coccinellae* and *O. scaposus* attacking immature stages of *H. axyridis* in Great Britain.

Material and methods

Pupae of *H. axyridis* and *C. septempunctata* were collected from sites in London and Cambridge, during the months of June to September, every year between 2004 and 2009. Host plants varied, but mostly included lime (*Tilia* spp.), sycamore (*Acer* spp.), rose (*Rosa* spp.), nettle (*Urtica* spp.) and tulip (*Liriodendron* spp.). Leaves bearing pupae were detached from the main plant, taken back to the laboratory and isolated into 5cm Petri dishes. Dishes were maintained at 22°C and constant light, and were kept under observation until adult eclosion or parasitoid emergence. Parasitism data for *C. septempunctata* is only available for the years 2004, 2008 and 2009. Rates of parasitism between years or between species were compared using two-by-two Fisher's exact tests.

Any adult coccinellids that eclosed were fed excess pea aphids, *Acyrthosiphon pisum* (Homoptera: Aphididae), for one week, and then transferred into 9cm Petri dishes in groups of eight individuals. These were supplied with an artificial diet (Majerus & Kearns, 1989), which was replaced twice a week. Several of the eclosed adult *H. axyridis* were later observed to give rise to the characteristic cocoons of *D. coccinellae*, which were isolated and allowed to eclose into adult wasps (Hall *et al.*, 2009).

Any phorid larvae that emerged from the ladybird pupae were allowed to pupate and eclose. Adults were identified as either *P. fasciata* or *P. berolinensis*, through microscopic examination using features described in Disney & Beuk (1997). Briefly, males of *P. fasciata* and *P. phalacrotphora* can be distinguished by inspection of the hypopygium: in *P. fasciata*, the right lobe of the hind margin of the hypandrium is clearly longer than the left lobe, whereas in *P. berolinensis* the lobes are of similar length. Female *P. berolinensis* are easily identified by the presence of dark thorn-like projections on each side of the terminal abdominal segment, which are lacking in *P. fasciata.*

Two pupae of *H. axyridis* produced both adult ladybirds and phorid larvae (Michie *et al.,* 2009). The phorid larvae were allowed to pupate and eclose, and were then identified as above. The ladybirds (both female) were weighed the day after eclosion, and kept on excess aphids until dead. They were then dissected under a stereomicroscope, and the condition of their fat body and the number of ovarioles in each ovary was noted. The weights and ovariole numbers of these parasitised individuals were compared to those from unparasitised individuals from the same cohort.

Some coccinellid pupae gave rise to broods of chalcid wasps emerging through one or two exit holes in the dorsal integument. The species is believed to be *Oomyzus scaposus*, based on the descriptions given in Ceryngier & Hodek (1996).

Results

From samples of *H. axyridis* pupae collected in 2004 (n = 278: all London), 2005 (n = 762: 682 London, 80 Cambridge) and 2006 (n = 450: 288 London, 162 Cambridge), no phorids were recovered. Two *H. axyridis* pupae collected in 2007, out of a Cambridge sample of 500 individuals, yielded broods of *P. fasciata* (0.4%). In 2008, a total of 1120 pupae were collected in Cambridge, and 18 of these gave rise to phorids (1.6%). This was a significant increase in infection rate (p = 0.049). Dissection of the phorids showed that sixteen of the pupae had been infected by *P. fasciata*, and two by *P. berolinensis.* No multiparasitism (parasitism by more than one species of parasitoid) was observed in this sample. Prevalence of infection of *H. axyridis* by phorids dropped to previous levels in 2009, with 11 of 2574 pupae (0.4%) being infected (2008-2009: $p < 0.001$; 2007-2009: $p \sim 1.00$). Dissection of 2009 phorid samples to determine species is still ongoing. Figure 1 shows the change in prevalence of phorid infection of *H. axyridis* between 2004 and 2009.

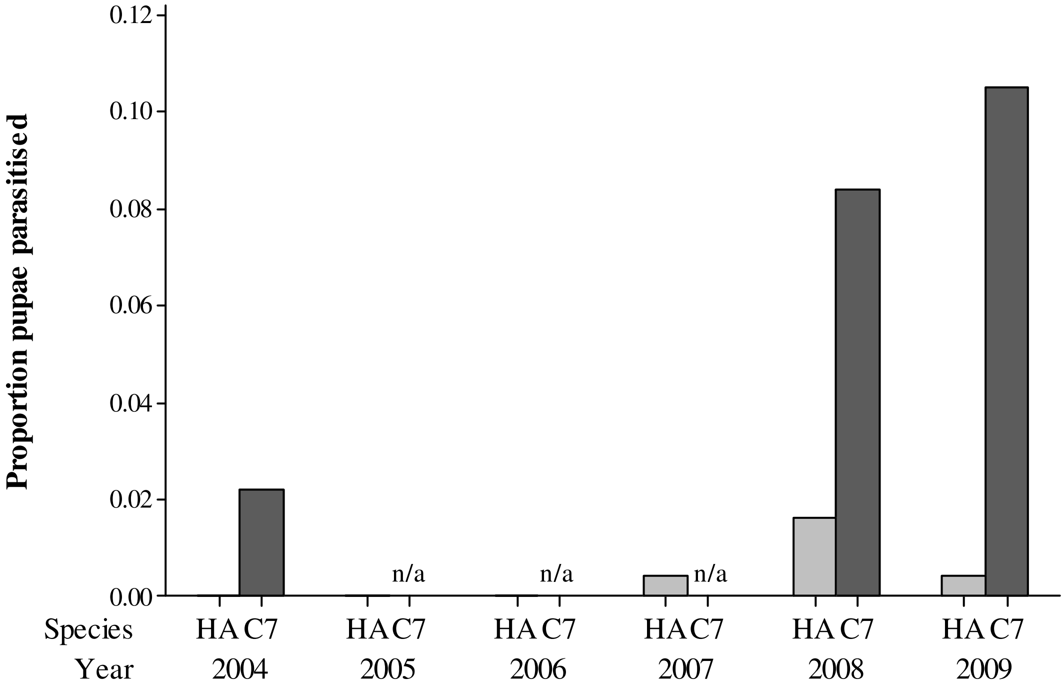

Figure 1. Prevalence of *Phalacrotophora* infection of *H. axyridis* (HA) and *C. septempunctata* (C7) between 2004 and 2009. 'n/a' indicates no data available for *C. septempunctata* in 2005, 2006 and 2007.

During this time period, the prevalence of phorid infection of *C. septempunctata* was significantly higher than that of *H. axyridis* ($p<0.001$), but itself increased from 2.2% in 2004 to 8.4% in 2008 ($p = 0.010$). There was no change in infection rate of *C. septempunctata* between 2008 and 2009 ($p = 0.623$). All phorids parasitizing *C. septempunctata* in 2004 and 2008 were identified as *P. fasciata.* Phorids recovered in 2009 are still to be identified.

Of the 1120 *H. axyridis* pupae collected in 2008, two produced a live female *H. axyridis* adult and one phorid larva each, as reported in Michie *et al.* (2009). Both phorids were identified as female *P. fasciata.* There was no difference in weight between the parasitized *H. axyridis* and unparasitsed individuals from the same cohort, but the fat bodies and ovariole counts appeared much reduced in the parasitised individuals (Michie *et al.,* 2009).

Of the successfully eclosed adult coccinellids in 2008, nine *H. axyridis* produced cocoons of *D. coccinellae*, as reported in Hall *et al.* (2009). However, only four of these cocoons gave rise to adult wasps, possibly due to predation by other ladybirds in the dish (R. Ware and R. Hall, pers. obs.). No adults of the other species collected were observed to give rise to *D. coccinellae.*

From the pupae collected in 2008, 2.5% of *C. septempunctata* were parasitized by *Oomyzus scaposus*, with broods of wasps emerging from one or two openings in the dorsal surface. No *H. axyridis* pupae were parasitised by chalcids in 2008. In 2009, thirteen out of 692 *C. septempunctata* pupae (1.8%) yielded *O. scaposus* broods, and two *H. axyridis* pupae out of 2574 (0.08%) were also infected.

Table 1 summarises the total parasitism data from this study.

Table 1. Parasitism of *H. axyridis* and *C. septempunctata* by three species of parasitoid in London and Cambridge between 2004 and 2009.

Year/ Species	Total pupae collected		Parasitism by *Phalacrotophora* spp.		Parasitism by *Dinocampus coccinellae*		Parasitism by *Oomyzus scaposus*	
	HA	C7	HA	C7	HA	C7	HA	C7
2004	278	227	0.000	0.022	0.00	0.00	0.00	0.00
2005	762	-	0.000	-	0.00	-	0.00	-
2006	450	-	0.000	-	0.00	-	0.00	-
2007	500	-	0.004	-	0.00	-	0.00	-
2008	1120	119	0.016	0.084	8 x10^{-3}	0.00	0.00	0.025
2009	2574	692	0.004	0.105	0.00	0.00	8 x10^{-4}	0.018

Discussion

This study documents the first observations of wild-infection of *H. axyridis* by the parasitoids *P. fasciata*, *P. berolinensis* and *O. scaposus* in Great Britain, and, to our knowledge, the first report of *D. coccinellae* successfully parasitizing *H. axyridis* larvae and/or pupae in the wild. Although it may be predicted that natural enemies occurring in Europe will eventually evolve novel adaptations that allow them to effectively utilise *H. axyridis* in addition to native species, the speed with which this seems to have occurred in Great Britain is remarkable. Within just five years of its arrival here, four of the major natural enemies of coccinellids in this country have begun to exploit *H. axyridis* as a novel host. The cosmopolitan nature of many of these enemies, particularly *D. coccinellae* and *Phalacrotophora* spp., offers at least some explanation, as does the fact that *H. axyridis* has been established in mainland Europe for a longer period, interacting with the natural enemies occurring there.

Clearly, it is difficult to tell whether the results reported here, from only a few years, truly represent the beginning of a co-evolutionary arms race between *H. axyridis* and British parasitoids. It is vital to continue monitoring these interactions over the next few years and decades, in order to evaluate the impact of parasitism on *H. axyridis* populations. Even with this limited dataset from the past five years, there have been some interesting fluctuations. For example, the rate of parasitisation of *H. axyridis* by phorids appeared to peak in 2008, followed by a significant decline in 2009. The difference between rates in 2008 and 2009 cannot obviously be explained by a reduction in phorid numbers in 2009, since parasitisation rate in *C. septempunctata* remained relatively constant during this period. However, the peak in 2008 occurred during a cold and wet summer in Britain, when aphid numbers plummeted (McCarthy, 2008, citing Rothamsted insect trap data). A tentative hypothesis for this observation could be that native ladybird species, such as *C. septempunctata*, suffered a population reduction in 2008, due to a combination of prey reduction and increased competition, and intraguild predation by *H. axyridis*. Meanwhile, *H. axyridis* may have suffered proportionately less due to its generalist diet and ability to capitalise on alternative prey items. If fewer *C. septempunctata* pupae were available as hosts for *Phalacrotophora* females, perhaps an increased rate of oviposition in *H. axyridis* was simply a result of relative densities. Although highly speculative, this is an interesting suggestion, since it could imply that the success of *H. axyridis* may eventually become limited by natural enemies that broaden their host range in the face of declining numbers of native host species.

A number of other interesting questions have been raised by this study which are worthy of further exploration. The ability of *D. coccinellae* to successfully parasitise coccinellid larvae and/or pupae is not unheard of, but occurs extremely rarely in the wild, and usually only when adult hosts are scarce (Geoghegan *et al.,* 1998). Indeed, choice tests performed in the laboratory indicate that female *D. coccinellae* show a strong preference to oviposit into adult ladybirds (Geoghegan *et al.*, 1998). The results shown here, and further documented in Hall *et al.* (2009), provide the first confirmation that *D. coccinellae* is ovipositing into pre-imaginal *H. axyridis* in the wild. It is widely accepted that adult *H. axyridis* are marginal hosts for *D. coccinellae* (Hoogendoorn & Heimpel, 2002; Berkvens *et al.,* 2009; Burling *et al.*, 2010), and, so far, parasitisation rates in Britain have been remarkably low (Koyama & Majerus, 2008). It will be interesting to continue monitoring relative parasitisation rates of both adult and pre-imaginal *H. axyridis* over the next few years. Although wasp development has been shown to take longer in less advanced developmental host stages (Berkvens *et al*., 2009), perhaps oviposition into larvae/ pupae allows the parasitoid to avoid immediate recognition and encapsulation by the host, which may constitute an effective defence mechanism in adults. The immune response of pre-imaginal stages to parasitisation by *D. coccinellae* is not currently well understood, and should be explored.

The survival of coccinellid hosts that have been parasitised by *Phalacrotophora* spp. is also not an entirely new phenomenon – it has been documented once before, in *Adalia bipunctata* (Majerus *et al*., 2000). Michie *et al.* (2009) suggest that the ability of *H. axyridis* to survive phorid infection could indicate some degree of host resistance, and this certainly warrants further investigation. In addition, they see the observation to be more widely significant in that it could provide a parasitoid-mediated route for the horizontal transmission of inherited symbionts, as also suggested by Majerus *et al.* (2000). In the case of *H. axyridis*, this is particularly interesting, as it harbours a male-killing *Spiroplasma* within its native Asian range (Majerus *et al.,* 1999) and, although male-killers have not yet been confirmed in British populations, its habitat overlaps with that of *A. bipunctata*, which is known to carry a male-killing *Rickettsia* in some populations. As phorids have now been recorded parasitizing both species of coccinellid, with host survival documented in each, transmission of bacteria between these species seems a possibility.

Further work into host-parasite morphometrics in the two gregarious parasitoid systems described here (*Phalacrotophora* spp. and *Oomyzus scaposus*) is already underway, with a view to assessing the relationships between parasitoid number, parasitoid size, host size and host species (R. Ware, unpubl. data). It is hoped that this analysis will shed some light on the suitability of *H. axyridis* as host to these parasitoids, in comparison to native host species. Future molecular genetic analysis will look for evidence of host specialisation within these species.

Overall, the interactions between *H. axyridis* and these parasitoids will provide a fascinating case-study of host-parasite co-evolution, and of the ecology of an invasive species in its new range.

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This paper is dedicated to the late Mike Majerus, who initiated this project in 2004 and collected many hundreds of ladybird pupae in London and Cambridge. The authors also wish to thank Henry Disney for advice on phorid dissection and identification, Ian Wright for technical support, and Peterhouse for fellowship funding.

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A feel for the organism: Ladybirding with Mike Majerus

Remy Ware
Department of Genetics, University of Cambridge, Downing Street, Cambridge, CB2 3EH, UK
E-mail: rlw29@cam.ac.uk

Michael Eugene Nicolas Majerus was born in London on the 13th February 1954, the middle child of three brothers. I think it is safe to say he was born an entomologist. His brother Paul recently said of Mike: 'Michael studied insects for 50 of the 54 years of his life. Therefore he considered the first four years to have been a waste'. In Mike's own words when giving an inspiring seminar in Sweden in 2007: 'I caught my first butterfly when I was four, and started recording the peppered moth when I was 10. I am getting old, and have spent my life in scientific enquiry and discovery. And it has been a great life!'

Mike studied botany and zoology at Royal Holloway College, London, graduating in 1975. He then embarked upon a PhD focusing on probably his favourite insect order, the Lepidoptera, looking at the ecology of the angleshades moth. Mike was then employed for a short while as a postdoctoral research demonstrator at the University of Keele, before being made a research associate in the Department of Genetics, University of Cambridge, in 1980. It was here that he conducted the rest of his academic life, making Lecturer in 1987, Reader in Evolution in 2001, and Professor of Evolution in 2007. Mike was also President of the Amateur Entomologists' Society, and a Life Fellow of the British Naturalists' Association.

Mike was internationally known in the fields of ecological and evolutionary genetics. He will be particularly remembered for his work on the evolutionary ecology of the Lepidoptera and Coccinellidae. His studies on defensive colour patterns and polymorphism in British moths and tropical butterflies will long be used to explain concepts of evolution by natural selection. A staunch defender of Darwinian evolution, Mike worked extensively with the 'peppered moth', *Biston betularia*, the celebrated example of natural selection in action, making a significant contribution to the evolution versus creation/ intelligent design debate. This was acknowledged internationally, with plenary or invited lectures at conferences in Australia, Canada, Japan, France, Italy and Sweden. His inspiring recent lecture, 'The Peppered Moth: The Proof of Darwinian Evolution', given at the European Society of Evolutionary Biology in Uppsala, Sweden, promoted significant discussion of the evidence for evolution.

During his work on ladybirds, Mike was the first to show that female mating preferences could be genetically determined, thereby confirming a critical aspect of Darwin's theory of sexual selection by female choice. He also worked extensively on the biased sex-ratios caused by 'male-killing bacteria' in some ladybird and butterfly species, showing that these endosymbionts can have significant evolutionary consequences for host populations, particularly with regard to male investment in mating and sex role reversal. In recent years, Mike's work also focused on the arrival of *Harmonia axyridis* in the UK. This subject proved popular with the British public, and thanks to numerous media appearances by Mike, thousands of records of the distribution of harlequin ladybirds (and native British ladybirds) were sent in from around the country, resulting in a dataset of unprecedented quality for the early establishment of an invasive species.

Mike worked tirelessly and passionately on the public dissemination of science, which was recognised in 2006 when he received the Sir Peter Scott Memorial Award for contributions to British natural history. The enthusiasm with which Mike communicated his ideas to both public and scientific audiences, coupled with his obvious skill and enjoyment of teaching, made him truly an inspiring individual. It is becoming increasingly rare to find biologists that meld the intricacies of genetics with what Barbara McKlintock called 'a feel for the organism', but Mike was one of the few who accomplished this synergy. Mike's exceptional research record resulted from a lifelong passion for his subject, his comprehensive knowledge of biological systems involving insects, and his unusual empathy for the habits of the organisms that he studied.

Mike was an incredibly charismatic individual, with boundless energy and optimism. It was often difficult to discern the boundaries between his life and his work: his work was his life, and one which he shared with all his family and friends. He often said he felt incredibly lucky to have made a living out of doing what he loved. Mike was happiest when he was trekking through some rainforest, or climbing up some mountain, as long as he had his butterfly net and a few collecting pots in tow! He took great pleasure in sharing these experiences with his family members and students, and you simply couldn't help but be fascinated by everything he said and did. Even when at home, he would be doing something entomological: pinning and setting his butterflies, putting out his moth trap, or making his garden insect-friendly. It is not often you meet someone whose love of life is so apparent: it is this that I will miss the most.

Mike was my mentor, and one of my closest friends. I met him during my first year as an undergraduate at Cambridge University, and embarked on a project with him in my second year, into ladybird cannibalism. Little did I know then that this was such an important life-changing move! There is no doubt that Mike has been the biggest inspiration in my life. He first enthused me about evolution and ecology, and helped me to develop a true love of natural history. After taking Zoology in my final year, I went on to do a PhD in Mike's group, and stayed with him ever since. I have so many fond memories of working with Mike, and I would have to say that the best of these relate to fieldwork. I got to travel all over the world with Mike, collecting ladybirds in Japan, Puerto Rico and across Europe. He was great fun to go ladybird-collecting (or has he called it, 'ladybirding') with. I always said he could spot a ladybird from 1000 paces, which he confirmed in 2004 when we visited a London park after hearing of the first record of *Harmonia axyridis* – he located a harlequin within 2 seconds of getting setting foot in the park! He assured me that one day I would be as good, and I remember him saying that he had truly corrupted me when I spotted a ladybird from a moving vehicle! But the most important thing Mike taught me was an attitude to scientific research and discovery. In the words of William Batason, Mike always encouraged me to 'treasure my exceptions' and indeed some of the most exciting parts of my research career so far have resulted from accidental discoveries and observations.

I had hoped that my close professional and personal relationship with this truly inspiring individual would continue long into the future. But Mike leaves a lasting legacy in his field, not only in terms of the valid scientific contributions he has made, but also in teaching and mentoring the evolutionary biologists of tomorrow. I am not alone in feeling that Mike changed my life: every student that has ever been lectured or supervised by him would agree that his infectious enthusiasm for the natural world left a lasting impression.

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Intraguild predation of non-coccinellid aphid natural enemies by *Harmonia axyridis*: prey range and factors influencing intraguild predation

Patricia M. Wells[1], Jason Baverstock[1], Michael E. N. Majerus[2], Frank M. Jiggins[2], Helen E. Roy[3] & Judith K. Pell[1]
[1]*Rothamsted Research, AL5 2JQ, UK;* [2]*University of Cambridge, CB2 3EH, UK;* [3]*Centre for Ecology and Hydrology, OX 8BB, UK.*
**Corresponding author: trish.wells@bbsrc.ac.uk*

Abstract: Although *Harmonia axyridis* has been recorded as an intraguild predator of various aphidophagous coccinellids, little is known about its interactions with other aphid natural enemies. We assessed the intraguild interactions between *H. axyridis* and four non coccinellid aphid natural enemies (two species of parasitoid plus two larval stages of lacewing). Petri dish trials showed that *H. axyridis* is an intraguild predator of 2nd instar lacewing (*Chrysoperla carnea*) larvae but not 3rd instar larvae. Predation of *Aphidius ervi* and *Praon volucre* parasitoid pupae was only observed occasionally. Experiments at a larger spatial scale and in more complex environments are needed to determine whether these interactions are ecologically important. The effect of alternative prey density and alternative prey species was assessed on whole plants in insectary cages. The density of alternative prey (pea aphid) did not affect predation of 2nd instar *C. carnea* by *H. axyridis* on bean plants. In contrast, aphid species may have an effect on aphid predation and intraguild predation.

Key words: intraguild predation, *Harmonia axyridis, Chrysoperla carnea, Aphidius ervi, Praon volucre*

Introduction

Harmonia axyridis is a recent addition to the aphid natural enemy guild in Britain (Brown *et al.*, 2008). This guild is made up of predators, parasitoids and pathogens and can provide an important ecosystem service via pest suppression in agriculture. Interactions between aphid enemies, in particular intraguild predation, can have important consequences for the structure of the community (Polis *et al.*, 1989). Intraguild predation between *H. axyridis* and other coccinellid species has received significant research attention in simple laboratory experiments (for example see Koch, 2003; Ware & Majerus, 2008), however, research is needed to determine the effect *H. axyridis* may have on non coccinellid guild members (Pell *et al.*, 2008).

Harmonia axyridis is a polyphagous coccinellid species with a prey range that includes non coccinellid aphid natural enemy species including predators (*Episyrphus balteatus, Metasyrphus corolla* and *Chrysoperla carnea*), parasitoids (*Aphidius ervi, Aphidius colemani*) and pathogens (*Pandora neoaphidis*) (Phoofolo & Obrycki, 1998; Takizawa *et al.*, 2000; Snyder & Ives, 2003; Gardiner & Landis, 2007; Roy *et al.*, 2008; Ware & Majerus, 2008; Putra *et al.*, 2009). Predation is affected by the behaviour of the predator and prey, the complexity of the habitat and the presence of alternative prey (Lucas *et al.*, 1998; Pell *et al.*, 2008). In particular, escape behaviour is one of the most common and effective defensive strategies of prey (Lucas *et al.*, 1998). These variables have a strong influence on intraguild prey survival in an agricultural landscape but cannot be included in simple laboratory arenas. Larger scale experiments using whole plants that allow the investigation of more natural behaviours in a more complex environment are needed to better understand intraguild interactions in an agricultural environment.

Previous studies have shown that the availability of alternative prey can reduce the level of intraguild predation by coccinellid predators (Sengonca & Frings, 1985; Lucas *et al.*, 1998; Nóia *et al.*, 2008), however, some studies have shown a lack of effect of alternative prey density on intraguild predation (Lucas *et al.*, 1998). It is not fully understood whether intraguild predation by *H. axyridis* is affected by the presence of alternative prey. In addition, the effect that alternative prey quality has on intraguild predation is not known. Adult *H. axyridis* females will preferentially predate on one aphid species over another (Soares *et al.*, 2004) and *H. axyridis* larvae will preferentially predate on aphid prey over parasitized prey (Snyder & Ives, 2003; Snyder *et al.*, 2004). *Harmonia axyridis* may not always preferentially predate on aphid prey in the presence of intraguild prey; certain aphid species may be less preferred compared to intraguild prey.

Here we present work assessing the susceptibility of lacewings *C. carnea* (second and third larval instars) and the parasitoids *A. ervi* and *Praon volucre* (both at the pupa stage) to predation by *H. axyridis* in simple laboratory arenas. Experiments done on whole plants investigating the effect of alternative prey density and alternative prey quality on the predation of *C. carnea* by *H. axyridis* are then reviewed. In addition, an experiment assessing the susceptibility of *Aglais urticae*, a lepidopteran species of public interest in Britain, is presented here.

Material and methods

Insects

Insect cultures were maintained at 18°C (LD 16L:8D). Two to eight week old adult *H. axyridis* from field collected *H. axyridis* (reared on *Acyrthosiphon pisum* maintained on *Vicia faba*) were used in experiments. *Chrysoperla carnea* larvae were purchased from Just Green (Crouch, UK) and Koppert UK Ltd. (Haverhill, UK). *Aphidius ervi* and *P. volucre* were maintained on *A. pisum* and one to three day old mummies were used in experiments. *A. urticae* larvae were purchased from Worldwide Butterflies (Cornwall, UK) and maintained on *Urtica dioica.*

Simple laboratory arena: determining the prey range of **Harmonia axyridis**

Experimental arena consisted of a 9cm Petri dish within which a single *H. axyridis* adult was offered six prey items. The prey treatments were: six potential intraguild prey of a single species (test prey), six adult *A. pisum* (control) or three test plus three control prey items. Male and female *H. axyridis* were tested for each prey treatment. Prey mortality in the absence of *H. axyridis* was also assessed. There were seven treatments in total for each intraguild prey species. The number of prey items consumed after 180 minutes was recorded. Each potential prey species was tested separately over four occasions.

To test the susceptibility of *A. urticae* to predation by *H. axyridis* the method was slightly altered with a single *A. urticae* larva (1-2cm in length) offered as prey to a fourth instar *H. axyridis* larva.

Complex laboratory arena: determining the variables affecting IGP

Complex laboratory arenas consisted of one plant pot containing four 18 to 21 day old *V. faba* plants held in a Perspex frame in an insectary cage (50x50x75cm). The soil at the base of the plants was covered with filter paper and the Perspex frames were flush with the top of the plant pot to create a sealed experimental arena. Where required plants were infested with aphids 24 hours prior to the start of the experiment. Second instar *C. carnea* larvae were released 30 minutes before the start of the experiment whilst female *H. axyridis* adults were released immediately prior to the start of the experiment. The arenas were maintained at 18°C (16L:8D) for 24 hours after which time *H. axyridis* adults were removed and the number of aphids and *C. carnea* remaining in each arena was counted.

Two experiments were done. The first experiment assessed the effect of aphid density on the predation of *C. carnea* by *H. axyridis*. Experimental arenas were infested with zero, 50, 200 or 500 three day old *A. pisum* nymphs. Ten *C. carnea* larvae were released into each arena and a single *H. axyridis* female was added to each arena for half the treatments (eight treatments in total).

The second experiment assessed the effect of alternative prey quality on the predation of *C. carnea* by *H. axyridis.* Predation of *C. carnea* was recorded in the absence of aphids or in the presence of either *A. pisum, Aphis fabae* or *Megoura viciae.* These aphid species had been maintained on *V. faba* and were predicted to vary in their palatability to *H. axyridis* (Hukusima & Kamei, 1970; Tsaganou *et al.*, 2004). Aphid size was controlled for by using instars of a similar weight. Fifty nymphs of each species were added to bean plants in each arena. The following treatments were set up for each aphid species: no enemy (control), ten *C. carnea* larvae, one adult female *H. axyridis,* and ten *C. carnea* plus one *H. axyridis*. In addition, two treatments without aphids were done: containing either ten *C. carnea* or ten *C. carnea* plus one *H. axyridis*. The 14 treatments were done on eight occasions in a randomised block design.

Results

Simple laboratory arena: determining the prey range of **Harmonia axyridis**

There was no difference in the number of second instar *C. carnea* larvae and *A. pisum* predated by *H. axyridis.* However, fewer third instar *C. carnea* larvae than *A. pisum* were predated by *H. axyridis*. More *A. pisum* were predated by *H. axyridis* when compared to the number of *A. ervi* and *P. volucre* predated. Similar results for all species tested were found when *H. axyridis* was offered test prey and aphid prey within a single arena. Fourth instar *H. axyridis* predated on *A. urticae* larvae in 63% of the replicates.

Complex laboratory arena: determining the variables affecting IGP

No effect of aphid density on the predation of *C. carnea* was found, however, intraguild predation by *H. axyridis* reduced the number of *C. carnea* larvae recovered (Table 1). A mean of 58% of the *C. carnea* larvae were recovered in the absence of *H. axyridis* and a mean of 38% were recovered in the presence of *H. axyridis.*

Table 1. Level of *C. carnea* predation due to cannibalism *(C. carnea* treatment), or cannibalism and intraguild predation (*C. carnea* + *H. axyridis*). Predation levels are on a scale of 1 (low predation) to 5 (very high predation).

Aphid treatment	*C. carnea*	*C. carnea* + *H. axyridis*
0	2	4
50	2	4
200	2	4
500	2	4

Chrysoperla carnea larvae did not differ in predation towards the three aphid species, however, *H. axyridis* predated more *A. pisum* and *A. fabae* than *M. viciae* (Table 2). When introduced together the predators consumed the most *A. fabae* and *A. pisum* and the least *M. viciae*, however, suppression did not seem to equal the sum of the level achieved by each predator species alone (Table 2). Cannibalism between *C. carnea* larvae did not differ with aphid species (Table 3). However, intraguild predation appeared to be affected by aphid species, with the most intraguild predation occurring with *A. pisum*, followed by *A. fabae* and *M. viciae.*

Table 2. Level of *A. pisum, A. fabae* or *M. viciae* predation by either ten *C. carnea* larvae, one *H. axyridis* adult, or ten *C. carnea* plus one *H. axyridis.* Predation levels are on a scale of 1 (low predation) to 5 (very high predation).

Aphid treatment	*C. carnea*	*H. axyridis*	*C. carnea* + *H. axyridis*
A. pisum	2	4	4
A. fabae	2	3	4
M. viciae	2	1	2

Table 3. Level of *C. carnea* predation due to cannibalism (*C. carnea* treatment), and intraguild predation and cannibalism (*C. carnea* + *H. axyridis*). Predation levels are on a scale of 1 (low predation) to 5 (very high predation).

Aphid treatment	*C. carnea*	*C. carnea* + *H. axyridis*
No aphid	3	5
A. pisum	3	5
A. fabae	3	4
M. viciae	3	3

Discussion

Previous studies have shown that *H. axyridis* is a predator of aphid parasitoids developing within aphids. *Harmonia axyridis* larvae predate on the pupae of *A. ervi* and *Aphelinus asychis* but preferentially predate on aphid prey (Snyder & Ives, 2003; Snyder *et al.*, 2004). However, adult *H. axyridis* beetles do not discriminate between aphid prey and aphids parasitized with *A. asychis* (Snyder *et al.*, 2004). In addition, *H. axyridis* can complete development on the larvae and the pupae of the aphid parasitoid *A. colemani* (Takizawa *et al.*, 2000). *Harmonia axyridis* is not the only coccinellid that can predate on developing aphid parasitoids. The coccinellids *Coccinella septempunctata* and *Propylea japonica* are also able to complete development on the larvae and pupae of *A. colemani*, although parasitoid pupae are not an optimal food source (Takizawa *et al.*, 2000). The larvae of *Coccinella undecimpunctata* predate developing *A. colemani* when parasitized aphid populations are high (Bilu & Coll, 2007). In addition, the presence of *C. undecimpunctata* had a negative effect on adult parasitoid behaviour which lead to a short term localised reduction in parasitism (Bilu & Coll, 2007). *Cycloneda sanguinea* reduced levels of the parasitoid *Aphidius floridaensis* through intraguild predation in field cage studies (Ferguson & Stiling, 1996).

In the current study adult *H. axyridis* predated second instar but not third instar *C. carnea* larvae. Gardiner and Landis (2006) have shown that second instar *C. carnea* can be predated by *H. axyridis.* In addition, Phoofolo and Obrycki (1998) have shown that *H. axyridis* and *Coleomegilla maculata* can complete development on a diet of *C. carnea* larvae. However, results from the current study show that larger larval instars of *C. carnea* are not vulnerable to predation from *H. axyridis* suggesting that *H. axyridis* may not have as big an effect on *C. carnea* as previously believed. The intraguild predation of *C. carnea* by coccinellids is not confined to the species *H. axyridis,* the larvae of *C. maculata lengi* and *C. septempunctata* will predate *C. carnea* larvae when the coccinellid larvae have a size advantage but *C. carnea* is the dominant predator in confrontations where size is more equal (Sengonca & Frings, 1985; Lucas *et al.*, 1998). Research is required to determine whether *H. axyridis* larvae have a similar size dependent relationship with *C. carnea* larvae.

Previous studies have shown that an increase in alternative prey density resulted in a reduction in intraguild predation by coccinellids in small laboratory arenas (Sengonca & Frings, 1985; Lucas *et al.*, 1998; Nóia *et al.*, 2008). Interestingly, Nóia *et al.* (2008) found that the level of intraguild predation by *H. axyridis* on *C. undecimpunctata* was less affected by an increase in alternative prey than the intraguild predation by *C. undecimpunctata* on *H. axyridis*. In the current study, *A. pisum* density did not affect the level of intraguild predation by *H. axyridis.* A similar result was found by Lucas *et al.* (1998); intraguild predation remained constant between first larval instars of *Chrysoperla rufilabris* and *C. maculata lengi* and between *C. rufilabris* and the gall midge *Aphidoletes aphidimyza* regardless of aphid density in experiments on two potato leaves (Lucas *et al.*, 1998). The authors suggest three (non-exclusive) reasons for a lack of effect of aphid density: the intraguild predator faced no risk in encountering the intraguild prey, foraging increases encounter rate between guild members and/or the concentration of the shared resource increased the risk of predator confrontation. Findings from the current study support these explanations; adult *H. axyridis* faced no risk from *C. carnea* and both predators use similar foraging strategies (Frazer, 1988).

The effect of alternative prey quality on intraguild predation was tested using three aphid species that are common in *V. faba* crops in Britain. It was found that *C. carnea* did not show a preference for aphid species whereas *H. axyridis* did show a preference for *A. pisum* and a dislike for *M. viciae. Harmonia axyridis* can complete development on *A. pisum* but cannot complete development on *M. viciae* (Tsaganou *et al.*, 2004). *Megoura viciae* has been reported to be toxic to other coccinellid species (Radwan & Lovei, 1983) but the cause of this is not yet known (Tsaganou *et al.*, 2004). Aphid numbers were lowest in the presence of both *H. axyridis* and *C. carnea* but this aphid suppression did not equal the sum of the level achieved by each predator species separately. Interference between the predators disrupted aphid suppression. Intraguild predation of *C. carnea* by *H. axyridis* has been shown to release aphids from control that would otherwise have been achieved by *C. carnea* in isolation (Gardiner & Landis, 2007).

From the current study it appears that aphid quality does have an effect on intraguild predation, the most preferred aphid prey resulted in the highest level of intraguild predation whereas the least preferred aphid prey resulted in the lowest level of intraguild predation. These results are surprising and give rise to two hypotheses. 1) The presence of preferred aphid prey stimulated predation, including intraguild predation, whereas the presence of the least preferred prey suppressed predation. In a previous field study, colonies of *A. pisum* tended to exist more often in the presence of *M. viciae* than would be expected by chance (Veen *et al.*, 2009). Furthermore, when predators were present, *A. pisum* colonies persisted for longer on plants with *M. viciae* than when alone. However, when predators were excluded, this difference in *A. pisum* colony survival was lost (Veen *et al.*, 2009). The authors suggest that *M. viciae* may be affecting the searching behaviour of natural enemies, possibly indirectly via the induction or inhibition of plant volatiles (Veen *et al.*, 2009). In the current experiment adult *H. axyridis* were placed at the base of plants containing *M. viciae* and *C. carnea*. The experiment started, in effect, after *H. axyridis* began intensively searching on a plant. The results from our experiments suggest that the inhibiting behaviour of *M. viciae* extends beyond plant selection to within plant foraging 2) Alternatively, aphid prey consumed by *C. carnea* larvae during the experiment may have affected the palatability of the *C. carnea* larvae to *H. axyridis.* Insects may become unpalatable after consuming certain prey. The cabbage aphid, *Brevicoryne brassicae*, sequesters glucosinulates from its host plant which are thought to aid in protecting the aphid against the predator *Adalia bipunctata* (Francis *et al.*, 2001). Host plants have been shown to affect the palatability of aphids to *H. axyridis*. The aphid *Aphis craccivora* collected from two legume species (*Vicia sativa* and *Vigna catiang* var. *sinensis*) was a good food source for *H. axyridis* whereas *A. craccivora* collected from three other legume and three brassica species (*Robinia pseudo-acacia, Astragalus sinicus, Vicia hirsute, Capsella bursa-pastoris, Cardamine flexuosa, Rorippa palustris* and *Hemistepta carthamoides*) was toxic to *H. axyridis* (Hukusima & Kamei, 1970). It is therefore possible that the palatability of *C. carnea* larvae was altered by the aphid species consumed during the experiment.

In conclusion, second instar *C. carnea* larvae may be at risk of predation from *H. axyridis* whereas third instar *C. carnea* as well as the pupae of *A. ervi* and *P. volucre* are not at risk of predation from *H. axyridis*. In addition, the extraguild prey *A. urticae* is at risk of predation from *H. axyridis*. The density of *A. pisum* was not found to affect intraguild predation of *C. carnea* by *H. axyridis*, however, aphid quality does appear to affect intraguild predation by *H. axyridis*, in particular *M. viciae* seems to reduce intraguild predation. The cause of this effect will now be investigated.

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PCR-based gut content analysis in *Harmonia axyridis*

[1]Renate Zindel*, [2]Angelos Katsanis, [2]Marc Kenis & [1]Alexandre Aebi
[1]*Agroscope Reckenholz-Tänikon ART Research Station, Reckenholzstr. 191, 8046 Zürich, Switzerland;* [2]*CABI Europe–Switzerland, Rue des Grillons 1, 2800 Delémont, Switzerland*
**Corresponding author: renate.zindel@art.admin.ch*

Intraguild predation (IGP) among ladybird species has been shown on several occasions (Agarwala and Dixon, 1992; Yasuda and Shinya, 1997). *Harmonia axyridis* is a voracious insect predator capable of devouring other ladybirds in experimental settings such as Petri dish arenas and field cages (Yasuda *et al*., 2004; Ware and Majerus, 2008). IGP is thus believed to be a key factor in the invasion success of *H. axyridis*. First, *H. axyridis* can eliminate competitors by IGP. Second, the consumption of intraguild species might contribute substantially to the diet for the larvae of *H. axyridis* (Snyder *et al.,* 2004). However, field observations of IGP remain scarce. Qualitative and quantitative data on intra-specific interactions among coccinellids are crucial to assess the importance of IGP in the apparent reduction of native ladybird populations observed in many parts of the world. Ecological data, experimental assays and chemical analyses showed that the species most at risk in Switzerland are *Adalia bipunctata*, *Adalia decempunctata*, *Calvia decemguttata* and *Oenopia conglobata* (Kenis *et al.,* 2010).

Harmonia axyridis, Adalia bipunctata and *A. decempunctata* have similar habitat preferences (Adriaens *et al*., 2007). Laboratory competition experiments have shown that both *Adalia* species rarely survive an encounter with *H. axyridis* in a Petri dish. However, the occurrence of IGP in the field remains to be demonstrated and quantified. Recently, a gut-content analysis technique based on the detection of exogenous alkaloids by GC-MS was developed and used to evaluate IGP of *H. axyridis* (Hautier *et al*., 2008). The technique was successfully applied to detect IGP in the field (Hautier *et al*., 2010). However, it failed to distinguish the two congeneric *Adalia* species.

In this study, we are developing molecular tools capable of a) detecting ladybird remains in the gut of *H. axyridis,* b) identifying the ladybird remains to species level and c) evaluating the importance of IGP at the ladybird community level in natural conditions.

We used published cytochrome oxidase I (COI) sequence information of *A. bipunctata*, *A. decempunctata*, and *H. axyridis* to design species –specific primers. The amplified region consists of a short section (ca. 100 bp) of the COI gene. We are developing a series of experiments and tests, confirming specificity, applicability and usefulness of the method. Digestion curves (evolution of detectability over time) will be elaborated for the four endangered species, analyzing the gut content of *H. axyridis* larvae having preyed upon coccinellids at defined time points after ingestion.

We believe that the molecular gut-content barcode system applied to *H. axyridis* will reveal previously unseen aspects of IGP in ladybird communities, shed light on the invasion success of *H. axyridis* and provide valuable information on the potential of such molecular tools for the development of comprehensive environmental risk assessment procedures.

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Is *Harmonia axyridis* a potential biocontrol agent in Christmas tree plantations?

Susanne Harding[1]*, Tove Steenberg[2]
[1]Dept. of Agriculture and Ecology, University of Copenhagen, Thorvaldsensvej 40, DK 1871-Frederiksberg C., Denmark; [2]Dept. of Integrated Pest Management, University of Aarhus, Skovbrynet 14, DK-2800 Lyngby, Denmark
**author for correspondence: suha@life.ku.dk*

Abstract: Nordmann fir (*Abies nordmanniana*) Christmas trees are a high value crop and quality demands are high. The adelgid *Dreyfusia nordmannianae* is the key pest in the production of Christmas trees, feeding on current-year foliage and causing needle distortion. Laboratory assays were carried out to evaluate the potential of *Harmonia axyridis* as a biocontrol agent in Christmas tree plantations. Differences appeared in the predation efficiency of larvae and adults as well as in the suitability as prey of different adelgid generations and developmental stages. *Harmonia axyridis* preferred and performed better on aphids belonging to Aphidoidea than on an adelgid diet. Our laboratory results indicate that *H. axyridis* may not be a highly effective biocontrol agent against *D. nordmannianae*. However, *H. axyridis* may assist in the natural regulation of the increasingly common lachnid *Cinara confinis* in Christmas tree plantations.

Key words: *Abies nordmanniana*, adelgids, biocontrol, Christmas trees, *Cinara confinis*, *Dreyfusia nordmannianae*, *Harmonia axyridis*, predation, survival

Introduction

Nordmann fir (*Abies nordmanniana*) is an economically important tree species in Danish forestry due to its widespread use as Christmas trees and decorative greenery. Quality demands are high and no insect damage is tolerated. *Abies nordmanniana* is attacked by an array of insect pests among which the silver fir adelgid, *Dreyfusia nordmannianae* is the key pest in the production of Christmas trees. *Dreyfusia nordmannianae* feeds on current-year foliage and on shoot axes, causing needle distortion and yellowing of the needles (Stephan, 1972). In addition, shoot extension is stunted and dieback may occur.

Dreyfusia nordmannianae may, along with other aphid species, reduce product quality severely and pesticides are regularly used to prevent damage (Larsen, 1997). Use of natural enemies may be an alternative means of reducing the extent of aphid problems. The 7-spot ladybird, *Coccinella septempunctata*, has been found to be a predator of *D. nordmannianae* (Ravn *et al.*, 2004), and the recent establishment of the harlequin ladybird, *Harmonia axyridis*, in Denmark (Steenberg & Harding, 2008) could be beneficial in providing natural regulation of aphids in Christmas tree plantations as it adapts to this habitat.

In Denmark, *D. nordmannianae* is almost exclusively anholocyclic. It overwinters in the hiemosistens stage at needles bases. By the end of March – beginning of April the hiemosistentes become active and lay clusters of eggs on the shoot axes. Egg-hatching coincides with bud burst and the neonate nymphs move to the new needles (progredientes ("crawlers")) or shoot axes (sistentes) to feed. The progredientes lay eggs on the needles, and the minute nymphs of this generation move to the shoot axis, where they settle and become inactive. Development is resumed in autumn prior to overwintering. Damage occurs in spring when progredientes and sistentes feed on the newly burst foliage (Stephan, 1972).

Only very few studies have described the activity of *H. axyridis* as a predator of Adelgidae (Butin *et al.*, 2004; Flowers *et al.*, 2006) and no studies have been carried out on the suitability of *D. nordmannianae* as prey of *H. axyridis*. We here report results of laboratory studies of the predation, survival and development of *H. axyridis* on *D. nordmannianae* to evaluate its potential as a biocontrol agent in Christmas tree plantations.

Material and methods

Predators

Adults and last instar larvae of *H. axyridis* were field collected and maintained in the laboratory at 20°C on a diet of *Sitobion avenae* reared on wheat. Neonate larvae were obtained from egg clusters sampled in the field and kept in the laboratory until hatching.

Predation

The predation of *H. axyridis* adults and larvae on different developmental stages and generations of *D. nordmannianae* was assessed in no-choice tests in 9cm Petri dishes.

Predators were starved for 24 hours prior to the assays. Adelgid prey was supplied as shoots of *A. nordmanniana* infested with *D. nordmannianae*. If more than one adelgid generation was present on the foliage, individual specimens or infested parts of the shoot were removed to ensure identical prey. One predator was added to each petri dish. The number of prey surviving after 1 hour, (4 hours), and 24 hours was recorded. Due to the dispersing behaviour of newly hatched progredientes, adult predation on this prey was recorded after 30 min and 1 hour. The experiments were conducted at 20°C. Ten replicates were completed per combination of predator and prey.

Predator survival and development

The survival and development of *H. axyridis* on different aphid prey was recorded in cage experiments.

The survival of *H. axyridis* adults feeding on the egg-laying progrediens generation on the needles and the sistens generation on the shoot axis was examined in 30x30x60cm ventilated plastic cages. Ladybirds were caged in groups of ten on branches of *A. Nordmanniana* infested with either of the two adelgid generations. Foliage was changed every second day to ensure ample and fresh food supply. Survival was recorded on day 2, 4 and 6.

Survival and development of *H. axyridis* larvae on different aphid prey was studied in 0.5l plastic containers with ventilated lids. In each container, foliage with prey was placed in a 15ml glass vial with water. Newly emerged larvae were transferred singly to the foliage. Prey species were *D. nordmannianae* (egg-laying progredientes + sistentes), *Aphis fabae* (on *Cirsium arvense*), and *Microlophium carnosum* (on *Urtica dioica*). Foliage was changed every second day. The larvae were inspected every day and survival and development time until pupation was recorded. The experiment was conducted at 20°C with ten replicates.

Results

Predation

Adult *H. axyridis* preyed on hiemosistens eggs and on small progrediens nymphs (Fig. 1). However, the ladybirds displayed no attraction or searching behaviour towards the minute progrediens nymphs that were consumed only by accidental encounter.

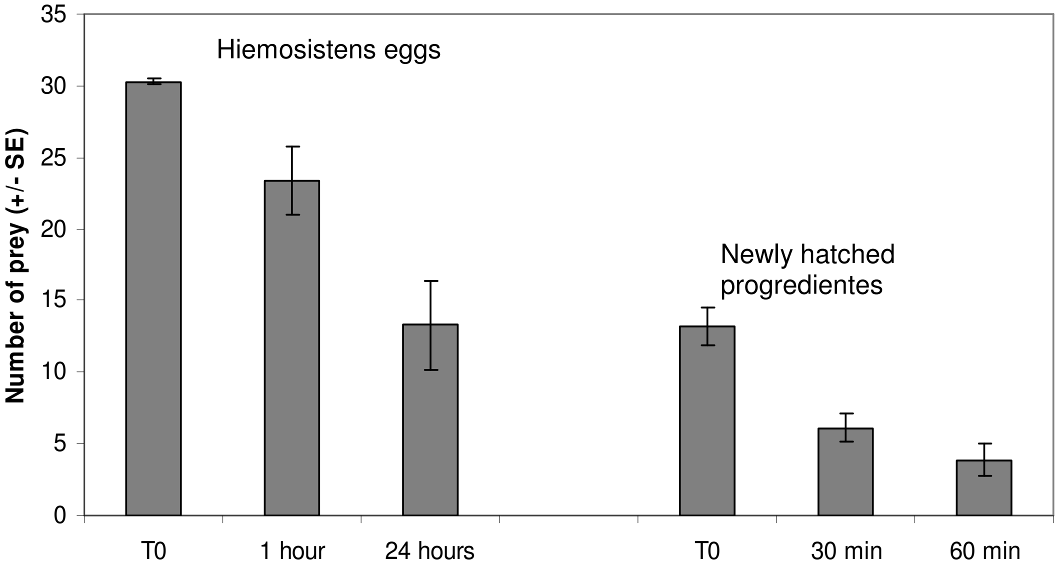

Figure 1. Predation of adult *Harmonia axyridis* on *Dreyfusia nordmannianae* hiemosistens eggs and minute progredientes ("crawlers") in no-choice tests in Petri dishes. Columns show number of prey surviving. *Harmonia axyridis* larvae were not tested as predators in this experiment, as they are not present in spring when hiemosistentes, eggs and newly hatched progredientes occur in the plantations.

Contrary to the minute crawlers, large (last instar) progrediens nymphs were readily recognized as prey by both adults and larvae of *H. axyridis* (Fig. 2). Also the egg-laying progredientes on the needles were preyed upon by adults and larvae of *H. axyridis*, although numbers eaten were lower than large progrediens nymphs (Fig. 3). Eggs were consumed before the progredientes.

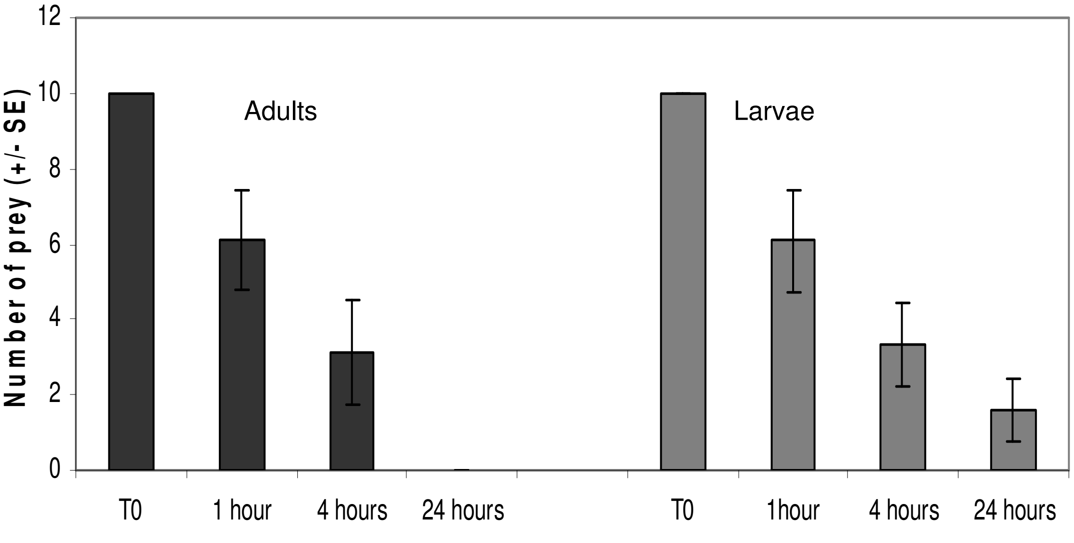

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Figure 2. Predation of *Harmonia axyridis* adults and larvae on last instar progrediens nymphs of *Dreyfusia nordmannianae* on needles of *Abies nordmanniana*. No-choice tests in Petri dishes. Columns show number of prey surviving after different time intervals.

The microscopic nymphs of the sistens generation on the shoot axis were not immediately recognized as prey (Fig. 4). After a period of further starvation in the petri dishes, moderate consumption of adelgids by adult as well as larval predators was observed. Numbers eaten were, however, low. For the larvae, predation caused mortality after 24 hours.

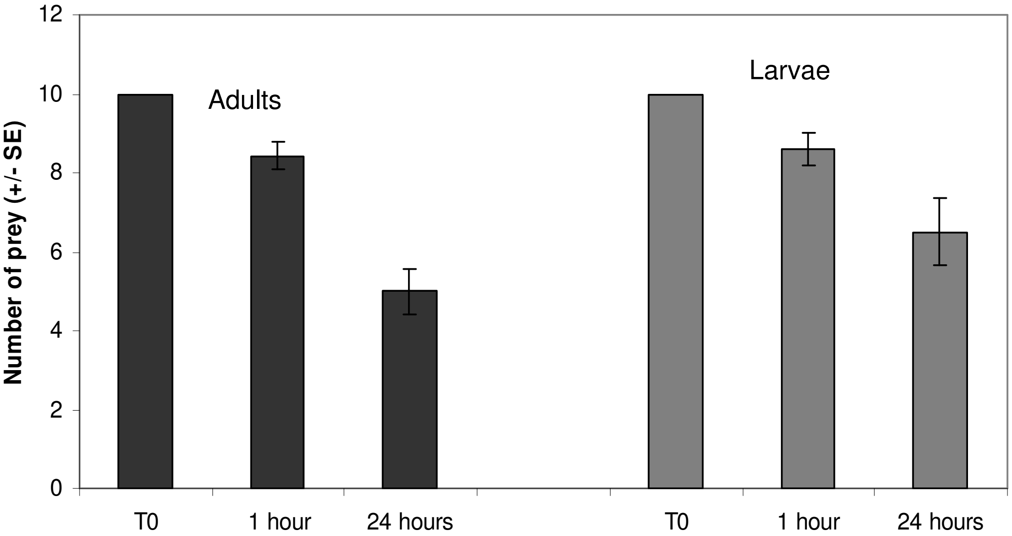

Figure 3. Predation of *Harmonia axyridis* adults and larvae on egg-laying progredientes of *Dreyfusia nordmannianae* on needles of *Abies nordmanniana*. No-choice tests in Petri dishes. Columns show number of prey surviving after different time intervals.

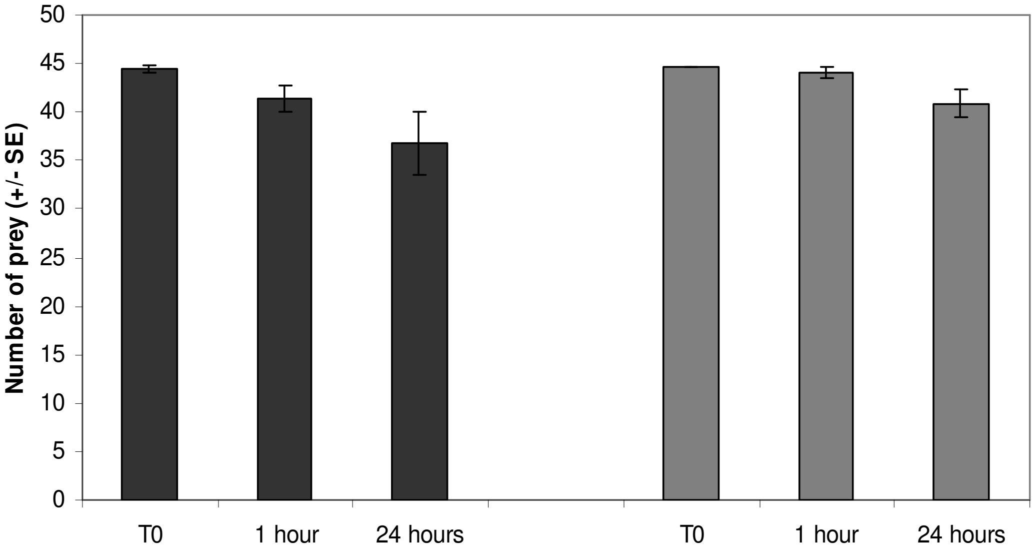

Figure 4. Predation of *Harmonia axyridis* adults and larvae on the minute, shoot-dwelling sistentes of *Dreyfusia nordmannianae* on shoots of *Abies nordmanniana*. No-choice tests in Petri dishes. Columns show number of prey surviving after different time intervals.

Predator survival and development

In the cage experiment, survival of adult *H. axyridis* was significantly lower on the minute shoot-dwelling sistentes than on the egg-laying progrediens generation on the needles (Fig. 5). When feeding on sistentes, predator mortality was observed already after 2 days.

No differences in survival were observed between neonate *H. axyridis* larvae transferred to foliage supporting different aphid prey. Development time until pupation, however, differed depending on prey species and was about 5 days longer on the adelgid *D. nordmannianae* than on aphids belonging to Aphidoidea (Fig. 6). Emergence of adults from pupae was successful on all prey species.

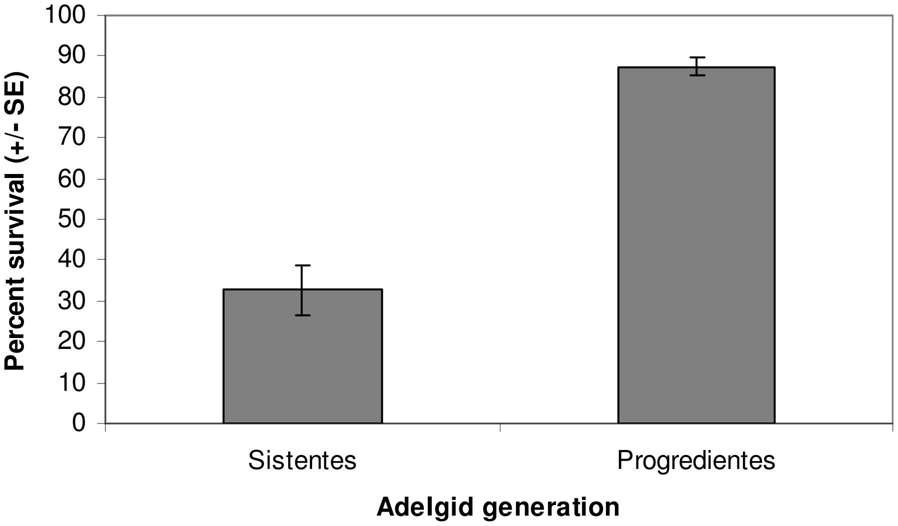

Figure 5. Survival of adult *Harmonia axyridis* feeding on sistentes and progredientes, respectively, of *Dreyfusia nordmannianae* in cage experiment.

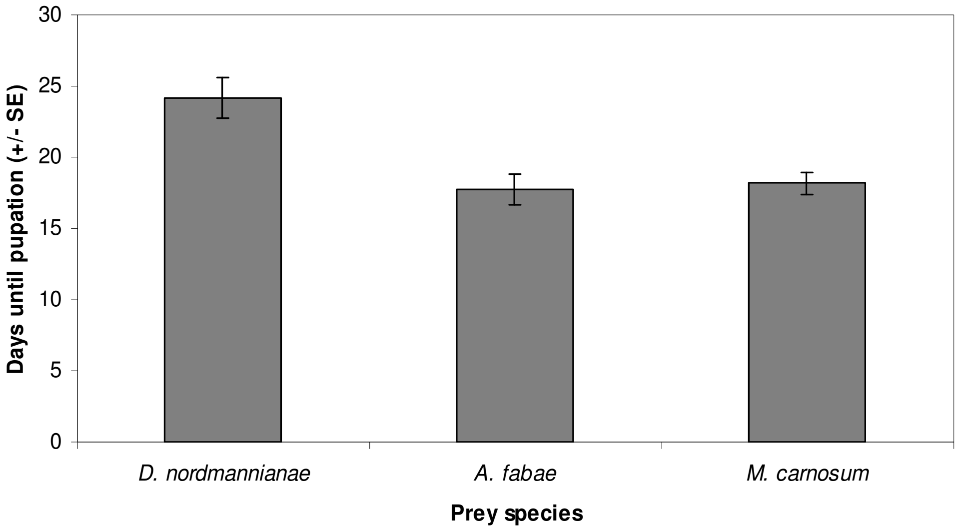

Figure 6. Development time from neonate larva until pupation of *Harmonia axyridis* on different aphid diet in cage experiments at 20°C.

Discussion

Harmonia axyridis was able to utilize the adelgid *D. nordmannianae* as prey, but significant differences appeared in the predation efficiency of larvae and adults as well as in the suitability as prey of different adelgid generations and developmental stages. Synchronization between the phenology of *H. axyridis* predators and different adelgid generations therefore may be crucial for the potential of *H. axyridis* to regulate *D. nordmannianae* populations in Christmas tree plantations.

By the time of spring activity and oviposition of hiemosistentes, *H. axyridis* is present in the Christmas tree plantations in the adult stage only, and even though our laboratory assessments show that adults do feed on hiemosistens eggs, the timing of emergence of overwintering *H. axyridis* in spring may be critical. *Harmonia axyridis* becomes active by late April (Steenberg & Harding, 2009), and their major feeding activity following emergence may in some years be too late to prevent adelgid population growth. Co-occurrence with the newly hatched progredientes and sistentes, which are causing the major damage on the foliage, is unlikely to lead to any significant reduction in adelgid numbers, since *H. axyridis* is not attracted to these stages and they are only preyed upon accidentally and to a limited extent. However, adult and larval predation on the egg-laying progredientes which are present on the needles in June may impact adelgid population levels.

Adult survival when feeding on progredientes was high, which is in accordance with observations on *H. axyridis* feeding on progredientes of the hemlock woolly adelgid, *Adelges tsugae*, in early summer (Flowers *et al.*, 2006). However, *A. nordmanniana* plantations can hardly support a *H. axyridis* population throughout summer, as adults and larvae of *H. axyridis* can not survive on a diet consisting exclusively of the shoot-dwelling sistens generation, which is the only adelgid generation present during mid summer to early autumn. Alternative prey is required and search for food may lead to migration of the adult *H. axyridis* to other habitats. In accordance, decreasing densities of *H. axyridis* in hemlock plantations infested by *A. tsugae* were recorded by Wallace & Hain (2000) and Butin *et al.*, (2004) in spite of high adelgid densities in summer.

It appears that adelgids are not a suitable diet for *H. axyridis* larvae: Butin *et al.* (2004) found that larvae were unable to complete development on *A. tsugae* and died during the early larval stage. In our experiment, *H. axyridis* development was completed, but significantly retarded when feeding on *D. nordmannianae* compared to a diet of non-adelgid aphids.

Harmonia axyridis may influence population levels of other aphid pests in Christmas tree plantations. In dual-choice tests with adult *H. axyridis*, aphids belonging to Aphidoidea were all preferred to *D. nordmannianae* (Harding, unpubl.). In contrast to the adelgids, these aphids were readily recognized as prey. The lachnid *Cinara confinis*, feeding on leaders and producing vast amounts of honeydew, is an aphid pest of increasing importance in Danish *A. nordmanniana* Christmas tree plantations (Harding *et al.*, 1998; Ravn, 2002). In semi-field experiments *H. axyridis* was attracted to Christmas trees infested by *C. confinis*, and searching behaviour and voracious predation was observed in laboratory (Harding, unpubl.).

In conclusion, our laboratory experiments indicate that *H. axyridis* may not be a highly effective biocontrol agent against *D. nordmannianae* in Christmas tree plantations. However, *H. axyridis* may perhaps assist in the natural regulation of the less important, but increasingly common *Cinara confinis*.

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