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Predation of native coccinellids by the invasive alien *Harmonia axyridis* (Coleoptera: Coccinellidae): detection in Britain by PCR-based gut analysis

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Abstract. 1. The harlequin ladybird, *Harmonia axyridis*, is an invasive alien species that arrived in Britain in 2003 and has spread across most of the country.

2. This spread has been accompanied by a decline in some native coccinellid species, in particular, the two spot ladybird *Adalia bipunctata* and the ten spot ladybird *Adalia decempunctata*. One reason for this decline could be intraguild predation (IGP) of the *Adalia* species by *H. axyridis*.

3. A DNA-based approach was used to look for evidence of coccinellid IGP in the wild. *Adalia bipunctata*- and *A. decempunctata*-specific Polymerase chain reaction primers were developed to analyse the gut contents of field collected *H. axyridis* larvae for the presence of *Adalia* DNA. *Harmonia axyridis* larvae (156) were collected from lime trees at four sites in eastern England over 3 years.

4. *Adalia bipunctata* DNA was detected in 7.7% and *A. decempunctata* DNA in 4.5% of *H. axyridis* larvae. DNA from both *Adalia* species was detected in two larvae.

5. This is the first demonstration of specific IGP interactions between *H. axyridis* and other coccinellid species in wild populations in Britain.

Key words. *Adalia bipunctata*, *Adalia decempunctata*, COI, gut contents, *Harmonia axyridis*, IGP, invasive species, PCR.

Introduction

The harlequin ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) is an invasive alien species in Europe that has spread at the rate of approximately 200 km per year over the last 10 years (Brown *et al*., 2011b). The species has become established in at least 26 European countries (Brown *et al*., 2011b) and has had negative impacts on several native ladybird species in England (Brown *et al*., 2011a), Belgium and Switzerland (Roy *et al*., 2012). *Harmonia axyridis* is a large, fast-breeding ladybird that is a generalist in terms of both habitat and diet (Koch, 2003; Berkvens *et al*., 2008 and references therein). While *H. axyridis* is primarily an aphidophagous species, the ephemeral nature of aphid populations means that at times it is likely to use alternative foods. Laboratory feeding trials have shown *H. axyridis* to feed on various insect groups (Koch, 2003; Sebolt & Landis, 2004; Ingels & De Clercq, 2010). These include the eggs, larvae and pupae of other ladybirds (Ware & Majerus, 2008), thus suggesting that *H. axyridis* exhibits intraguild predation (IGP) in the wild. IGP is the killing and eating of a species that uses similar, often limiting, resources (Polis *et al*., 1989). Because the prey animal is also a competitor, IGP differs from classical predation in that the predation event reduces potential exploitation competition (Polis *et al*., 1989). IGP is widespread in many trophic systems (Polis & Holt, 1992) including the guild of aphidophagous species that includes many ladybirds (Dixon, 2000). Intraguild predation by *H. axyridis* on other ladybird species may be commonplace (Gagnon *et al*., 2011b) and may therefore have the potential for highly negative effects on co-occurring populations of native ladybirds (Gardiner *et al*., 2011). Moreover, IGP is listed as an environmental risk to be considered before the release of beneficial arthropods for biological control (Aebi *et al*., 2011; Gibbs *et al*., 2011).

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Direct observation of predation events (including IGP) in the field is difficult and labour intensive, especially in the case of small arthropods, and may disturb the study system (Sunderland, 1988). Thus, over the years, alternative (indirect) methods of detecting predation have been developed. For example, predators may be field collected and analysed later in the laboratory. Gut contents may be analysed by microscopic observation of prey fragments, but this method is very time-consuming (Greenstone *et al*., 2007). In recent years molecular tools have provided opportunities for exploring predator-prey interactions in ecosystems. These tools include monoclonal antibody -(MAb) based assays, gas chromatography mass spectrometry (GC-MS) and increasingly, detection of prey DNA using the Polymerase Chain Reaction (PCR). In general, MAb has been displaced by PCR and GC-MS. PCR enables detection and identification of prey by analysis of the DNA present in the predator gut and has increasingly been used to assess predation in the field by a range of invertebrate taxa, including spiders (Agusti *et al*., 2003) and beetles (Pons, 2006). GC-MS has been used to detect IGP between coccinellid species (Hautier *et al*., 2011). It detects trace amounts of exogenous alkaloids in the predator gut. GC-MS, although highly sensitive, tends to be less specific than PCR-based methods (reviewed by Aebi *et al*., 2011).

In selecting an appropriate technique for detecting IGP, a major consideration is the detection time window, that is, for how long following prey consumption this predation can be detected (Aebi *et al*., 2011). Some assays involving GC-MS (e.g. for the alkaloid adaline) have a long detection window (Hautier *et al*., 2011). Prey detection time can be extremely variable between different PCR assays, ranging from a few hours to several days (King *et al*., 2008; Gagnon *et al*., 2011a). To make a meaningful ecological assessment of IGP, a balance needs to be struck in terms of prey detection time; too short a detection window would likely mean that the number of positives would be too small to extrapolate; but too long a detection window would make the ecological significance of the detection difficult to interpret: quantifying IGP is more difficult when the time span is longer (Fournier *et al*., 2006).

We chose to develop a PCR-based detection system which aimed to detect and quantify the level of IGP by *H. axyridis* on two native ladybird species, *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae) and *Adalia decempunctata* (L.) (Coleoptera: Coccinellidae), in wild ladybird populations in England. The two *Adalia* species were chosen because their niches overlap most closely with *H. axyridis*; these three species being the most abundant coccinellids found on the lime trees at our survey sites. In our study, we designed PCR primers for amplification of sequences within the mitochondrial cytochrome oxidase I gene (COI) and ITS1 region of *A. bipunctata* and *A. decempunctata*. The digestion rate of *Adalia* DNA within the gut of *H. axyridis* was assessed via a series of controlled feeding trials. Finally, the primers were used on DNA extracted from field collected *H. axyridis* larvae. Our study is novel in that these PCR detection techniques, once developed, have been applied to a field situation to address an area of conservation concern.

Materials and methods

Field collection of ladybirds

To assess the level of IGP by *H. axyridis* on *A. bipunctata* and *A. decempunctata*, third and fourth instar *H. axyridis* larvae were field collected by tree-beating lime trees, *Tilia* × *europaea* L. (Malvaceae), the dominant vegetation at four churchyard sites in East Anglia, England – St Ives (Ordnance Survey grid reference TL309716), Fordham (TL633707), Worlington (TL691738) and Wilburton (TL477749). Lime trees were chosen because in the study area they supported relatively high abundances of the study ladybird species. Branches were beaten with a stick above a 110 × 86 cm white canvas beating tray.

All sites were known in advance to have populations of *A. bipunctata*, *A. decempunctata* and *H. axyridis* (Brown *et al*., 2011a), thus the opportunity for IGP between these species existed. Ladybird collection took place in late June or early July 2008, 2009 and 2010. Aphidophagous ladybirds are generally diurnal feeders (Nakamuta, 1987), and 1000–1600 h was shown to be the main feeding window observed for *H. axyridis* in Japan (Miura & Nishimura, 1980). To maximise the chances of finding ladybirds that had recently fed, all surveys were carried out between 1000 and 1600 h, in dry weather, with air temperature at site of at least 17 °C. Ladybirds were placed individually in 1.5 ml Eppendorf tubes at the field site and immediately put on ice in an insulated box. They were thus alive but inactive, with very slow metabolism and digestion. Within 90 min, the collected samples were frozen at −20 °C, before being transferred to a −80 °C freezer for long-term storage.

DNA extraction

Each larva was placed in a separate 1.5 ml Eppendorf tube and crushed with a plastic micro-pestle. DNA was then extracted using a DNEasy Tissue Kit (Qiagen, UK) according to the manufacturer's instructions. The main stock DNA was frozen at −20 °C. A working aliquot was refrigerated at 4 °C. The presence of amplifiable DNA was tested with a PCR using a *H. axyridis* microsatellite primer pair, Ha281 (Loiseau *et al*., 2009).

Primer design

Detection of prey DNA in predator guts by a PCR-based system is dependent upon successful amplification of prey-specific undigested DNA fragments. Thus, it is best to target short (< 200 nucleotide) multi-copy DNA sequences. Two types of target sequences have proved informative. Sequences within mitochondrial genes provide multiple targets with hundreds to thousands of copies per insect cell (Hoy, 1994). The mitochondrial COI and COII genes have proved successful targets in various arthropod studies (reviewed by King *et al*., 2008 and Aebi *et al*., 2011). Multi-copy nuclear genes are alternative useful target sequences. A number of IGP studies have amplified sequences in the internal spacer regions of the nuclear ribosomal

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gene complex, for example, Hoogendoorn and Heimpel (2001, 2002).

Gene sequences for COI and ITS1 were found in Genbank for *A. bipunctata, A. decempunctata* and *H. axyridis*. The sequences for each gene were aligned using EMBI biotools (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Areas of dissimilar sequence were identified, and primers were designed by eye and checked using the software "Primer3" (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). We aimed for 60% GC content, but not runs of GC. Primers were then subjected to specificity and sensitivity checks as outlined below.

PCR amplification

Each PCR reaction (25 μl) contained 11.7 μl double distilled water, 10× amplification buffer (2.5 μl) (supplied by the manufacturer with the enzyme), 1.5 mM of $MgCl_2$, Q solution (4 μl), 100μM of each dNTP, 10 μM of each primer, 0.05 units of Taq (Qiagen) DNA Polymerase and 1 μl of extracted DNA. Amplification was performed in a TECHNE TC 412 thermocycler. The PCR cycle regime used for ITS1-AD2 was an initial denaturation of 94 °C for 3 min and then 35 cycles of denaturation at 94 °C, annealing at 53 °C, and extension at 72 °C (1 min for each stage). The final extension was 72 °C for 10 min. Touchdown PCRs were used for primers COI-Abip and COI-Adecem. For the COI-Abip touchdown PCR, the annealing temperature was decreased 1°C every two cycles from 63 to 59 °C, then maintained at 58 °C for 35 cycles. For COI-Adecem, annealing temperature was decreased 1 °C every cycle from 62 to 53 °C, then 30 cycles at 52 °C. PCR products were separated on a 2% agarose gel or 3% metaphor agarose gel for COI-Abip products. Gels contained 2 μl Ethidium Bromide (25 mg ml^{-1}), and were run at 70 volts for 2 h in 1% TBE buffer. All PCR products were then photographed on a UV transilluminator for analysis. Three controls were included in each IGP-amplification: a negative control containing DNA from a laboratory-reared *H. axyridis* which had been reared on an aphid only diet; a positive control of 100% prey DNA; and a reaction blank which contained distilled water instead of DNA.

Specificity and sensitivity of primers

Specificity tests. Once PCR conditions were optimised for a pair of primers, their specificity to amplify the prey species of interest was tested. Twelve individuals of each of *A. bipunctata, A. decempunctata* and *H. axyridis* were used in the specificity tests. For primers that were specific for a single *Adalia* species, cross reactivity was tested with four other coccinellid species (three individuals of each) that were also found in the sample sites, namely *Coccinella septempunctata* L. (Coleoptera: Coccinellidae), *Propylea quattuordecimpunctata* (L.) (Coleoptera: Coccinellidae), *Calvia quattuordecimguttata* (L.) (Coleoptera: Coccinellidae) and *Exochomus quadripustulatus* (L.) (Coleoptera: Coccinellidae). To confirm that the primers amplified the desired COI or ITS1 target, all positive result DNA bands from the field collected larvae were sequenced.

Sensitivity tests. To assess the sensitivity level of the primers, a series of tenfold dilutions of prey to *H. axyridis* DNA, that is, 1 part prey to 1 part *H. axyridis*, diluted to 1 part prey to 10^6 parts *H. axyridis* DNA, were used as templates in PCRs. The aim of this test was to assess the ability of a chosen primer pair to detect prey DNA within a mass of predator DNA, which represents, to some extent, a natural situation (Aebi *et al*., 2011).

Feeding trials

To establish a digestion curve of the target DNA region within the gut of *H. axyridis*, *A. bipunctata* eggs were presented to final (i.e. fourth) instar *H. axyridis* larvae in a series of feeding experiments. Final instar *H. axyridis* larvae and *A. bipunctata* eggs were used because these are the life cycle stages more probable to engage in, and suffer from IGP, respectively (Cottrell & Yeargan, 1998; Lucas *et al*., 1998). All ladybird stock was of British origin, laboratory-reared at the Department of Genetics, University of Cambridge or Rothamsted Research and fed on pea aphids, *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae). The larvae were starved for 24 h immediately prior to experimentation to increase their motivation to forage and consume the eggs.

Harmonia axyridis larvae were placed individually in clean, dry 2.5-cm-diameter Petri dishes containing five *A. bipunctata* eggs in a controlled laboratory environment at 21 °C with constant light. Larvae were left to feed under observation, and the feeding start-time (i.e. when a larva actually started consuming the eggs) and finish-time were recorded. Each larva was allowed to feed for a maximum of 30 min and the number of eggs eaten was recorded. After feeding, each larva was transferred individually to a clean Petri dish. Following the allocated digestion period (i.e. 0, 2, 4, 6, 8, 10, 12, 18, 24, 30 or 36 h), the larva was transferred from its Petri dish to a 1.5-ml Eppendorf tube and frozen, initially at −20 °C, before transfer on ice to longer-term storage at −80 °C, ready for DNA extraction for the PCR experiments at a later date. Five replicates were performed for each digestion period. This included five control *H. axyridis* larvae which were not fed *A. bipunctata* eggs.

Results

Specificity and sensitivity of primers

Three primer pairs were selected for use in this work. Each amplified a small DNA fragment sized between 80 and 110 base pairs (Table 1). The COI-Adecem primer pair was specific to *A. decempunctata,* while the COI-Abip primer pair, in addition to strongly amplifying *A. bipunctata* DNA, also occasionally weakly amplified *H. axyridis* DNA, producing a 70-base pair band. The 10 bp difference of the *A. bipunctata* and *H. axyridis* amplicons was clearly distinguishable when electrophoresis was performed through high resolving metaphor agarose (Fig. 1). The COI-Abip and COI-Adecem primers were extremely sensitive. They strongly detected *A. bipunctata* and *A. decempunctata*

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Table 1. Details of primers used for detection of *Adalia* species.

Primer name	Target	Sequence (5′-3′)	Product size (bp)
COI-Abip	COI	F: GAC CCA ATG GAT GAA ACC R: GGA TTA AGA GGA ATA CCA CGA C	80
COI-Adecem	COI	F: GGA TTA CTC CAG TTA AGC C R: GAC TTG CAA CAT TAC ACG G	105
ITS1-AD2	ITS1	F: CGT AGA GAA CGG GAT TCG TC R: TTA TGT TTG TGT TGT CTC ACG TC	99

COI, cytochrome oxidase I gene.

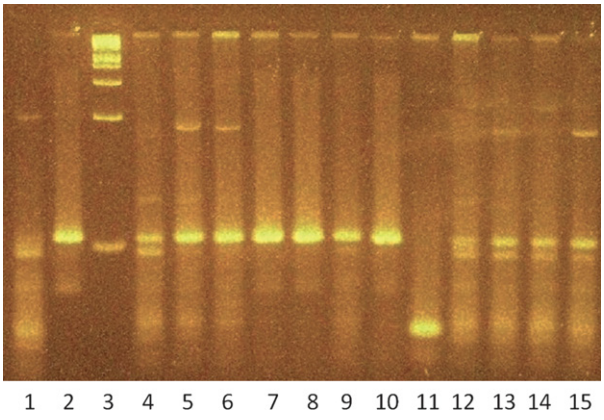

Fig. 1. Metaphor agarose gel electrophoresis (3%) of Polymerase Chain Reaction-amplified DNA using the cytochrome oxidase I gene-Abip primer pair. This gel shows the target *Adalia bipunctata* amplicon of 80 bp and the occasional *Harmonia axyridis* amplicon of 70 bp. Lane 1, *H. axyridis*; lane 2, *A. bipunctata*; lane 3, molecular size marker: hyperladder V: Bioline (bottom band 75 bp); lanes 4–15 *H. axyridis* larvae which had consumed *A. bipunctata*, with the exception of the larva in lane 11.

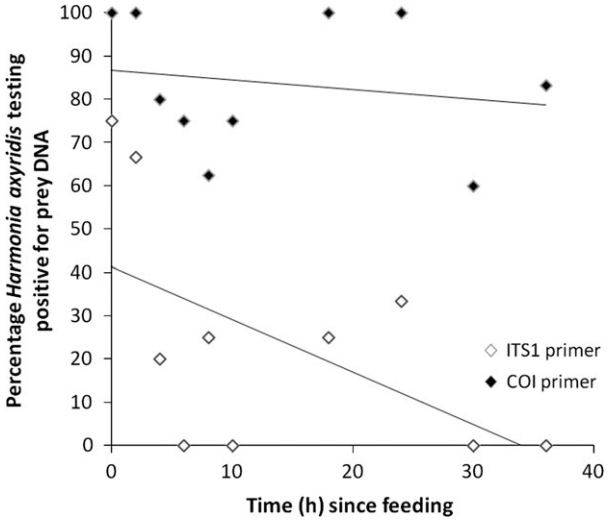

Fig. 2. Detection of *Adalia bipunctata* DNA in *Harmonia axyridis* larvae from feeding trials.

DNA, respectively, at a presence of 0.0001% (a mix of 1 part prey to 10^6 parts *H. axyridis* DNA).

In cross reactivity tests, all ITS1 primer pairs amplified both *Adalia* species. The ITS1-AD2 primer pair was highly specific to *Adalia* spp. and never amplified *H. axyridis* DNA or the four other coccinellid species tested for cross reactivity. The sensitivity of the ITS1-AD2 primer pair was low. These primers could not detect *Adalia* prey DNA below 1% (i.e. a mix of 1 part prey to 100 parts *H. axyridis* DNA). The 10^4 lower sensitivity of the ITS1-AD2 primer pair compared with the COI primer pairs, suggested the ITS1 pair could provide a useful monitor of recent IGP, that is, they would only detect *Adalia* DNA before extensive digestion had reduced it to low levels. Thus, the ITS1-AD2 primer pair was included in our IGP analysis.

Feeding trials

The ability of the PCR primer pairs COI-Abip and ITS1-AD2 to amplify prey DNA from *H. axyridis* larvae guts was assessed via controlled feeding trials, when DNA was extracted at varying times (Fig. 2) following ingestion of five *A. bipunctata* eggs. DNA was amplified successfully with both primer pairs. The COI-Abip primers showed frequent strong detection of *Adalia* DNA: prey DNA was detected in 78% of all larvae used in the feeding trial, including four of the five larvae sampled 36 h post-ingestion. In contrast, after 4 h post-ingestion (when *A. bipunctata* DNA was detected in one of the five replicates), the ITS-AD2 primers generally failed to detect *A. bipunctata* DNA.

PCR analysis of field collected Harmonia axyridis *larvae*

A total of 156 *H. axyridis* larvae were field collected and PCR tested for the two target prey species using the two COI primer pairs. Detection of *A. bipunctata* DNA within the gut contents of *H. axyridis* larvae was made at all four sites and in all 3 years. *Adalia decempunctata* DNA was detected within *H. axyridis* at three sites and in 3 years (2009 and 2010) (Table 2 and Fig. 3). The overall *Adalia* detection rate for the 3 years was 12.2% (i.e. 19 of 156 larvae). The rates of detection increased from 2008 to 2010. In 2008, just 3.7% of *H. axyridis* larvae revealed the presence of *A. bipunctata*. This increased to 12.0% of *H. axyridis* larvae collected in 2009 showing the presence of either *A. bipunctata* or *A. decempunctata* DNA and 22.7% of the field collected

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Table 2. Rates of detection of *Adalia bipunctata* and *Adalia decempunctata* DNA in *Harmonia axyridis* larvae collected from four field sites in East Anglia, England, over 3 years. (Total number of positive results is given at the top of each table section.)

	St. Ives	Fordham	Worlington	Wilburton
A. bipunctata – 12/156 (7.7%)				
2008	2/39	–	0/10	0/5
2009	1/11	3/22	0/5	1/20
2010	–	1/20	2/10	2/14
A. decempunctata – 7/156 (4.5%)				
2008	0/39	–	0/10	0/5
2009	0/11	0/22	1/5	1/20
2010	–	3/20	0/10	2/14
Total (*Adalia*) – 19/156 (12.2%)				
2008	2/39	–	0/10	0/5
2009	1/11	3/22	1/5	2/20
2010	–	4/20	2/10	4/14

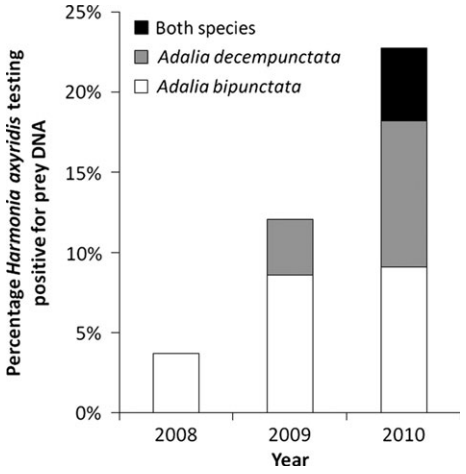

Fig. 3. Summary of rates of detection of *Adalia bipunctata* and *Adalia decempunctata* DNA in *Harmonia axyridis* larvae collected from four field sites in East Anglia, England, over 3 years. Bars show *A. bipunctata* only, *A. decempunctata* only and both species together.

larvae in 2010. In 2010, two *H. axyridis* larvae, from different sites (Fordham and Wilburton), showed the presence of DNA from both *Adalia* species.

Those *H. axyridis* larvae that amplified positively with the COI primer pairs were then tested with the ITS1-AD2 primer pair for an assessment of how recently before sampling the *Adalia* predation had occurred. Three larvae tested positively with the ITS primers. These were two larvae collected at Fordham in 2009 which had tested positively for the presence of *A. bipunctata* DNA and a third Fordham larva collected in 2010 which showed the presence of DNA from both *Adalia* species. This ITS indication of recent *Adalia* predation correlated with particularly strong PCR bands for these three larvae when initially tested with the COI-Abip primers. The ITS1-AD2 test showed that the three Fordham larvae in question are likely to have consumed their *Adalia* prey within 4 h prior to collection, as well as confirming *Adalia* detection.

Discussion

PCR-based analysis of the gut contents of *H. axyridis* revealed IGP of both *A. bipunctata* and *A. decempunctata* under field conditions in England. Furthermore, the level of IGP increased sixfold over the 3 years of our study, from 3.7% in 2008 to 22.7% in 2010. IGP was confined to *A. bipunctata* in the first year of the study. In subsequent years, predation of both *Adalia* species was detected (Fig. 3).

Coccinellid populations have been monitored from 2006 to 2008 at two of the four sites (St Ives and Fordham) sampled in the current study, encompassing the invasion phase of *H. axyridis* in Eastern England (Brown *et al.*, 2011a). During these 3 years, there were dramatic changes in the ladybird assemblages at these and other local sites. In 2006, 0.1% of the ladybirds sampled were *H. axyridis*. Two years later, 40% of the ladybirds were *H. axyridis*. This increase in *H. axyridis* over the 3 years was paralleled by declines in *A. bipunctata*, and to a lesser extent *A. decempunctata* (Brown *et al.*, 2011a). Positive detection of IGP at an average rate of around 12% indicates that IGP was one mechanism behind the observed declines in native species, and we suggest that it may have been the most important mechanism.

While we have observed increasing levels of IGP over the 3 year period of this study, with greater predation of *A. bipunctata*, it may be that further IGP increases are unsustainable. The availability of *Adalia* ladybirds to be preyed upon will clearly affect the IGP rate. This prey availability has reduced over time, *A. bipunctata* faster than *A. decempunctata*. In terms of the impact of absolute and relative abundance of *A. bipunctata* and *A. decempunctata* on rates of IGP, it is interesting to note that there was a 3.37-fold increase in IGP of *A. decempunctata* from 2009 to 2010, but only a slight increase (1.32-fold) in IGP of *A. bipunctata* between these 3 years. This implies that as IGP and other factors reduce the abundance of a species, IGP itself may reduce as new equilibrium frequencies of the different ladybird species are established.

The importance of IGP in terms of negative effects on *Adalia* species increases with increasing overlap in the timing of juvenile stages of predator and prey. At our study sites, predator and prey larvae co-occurred in approximately 50% of surveys (Brown *et al.*, 2011a), representing a high degree of overlap. The importance of IGP as a survival mechanism to *H. axyridis* will vary depending on the season. In England, *H. axyridis* appears usually to have two generations per year (Brown *et al.*, 2008), with second generation larvae not emerging until September or October. At this time of year, there are generally no eggs, larvae or pupae of other coccinellid species present, thus preventing IGP. Second generation *H. axyridis* also rely on other food sources, including cannibalism (Osawa, 2011), for their development.

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In this study, we did not test the reverse IGP scenario, that is, the possibility that *H. axyridis* was being predated by *Adalia* species. *Harmonia axyridis* larvae and adults have a size advantage over *Adalia* species (and most other native coccinellids in England) (Roy *et al*., 2011), a trait that is very important in determining the outcome of an IGP encounter (Ware & Majerus, 2008). Additionally, *H. axyridis* is well defended both physically (e.g. larvae possessing thick dorsal spines) (Ware & Majerus, 2008) and chemically (Sato & Dixon, 2004). Together, these traits suggest that this reverse IGP scenario is unlikely to be important; indeed, in laboratory trials, interactions between coccinellid larvae were asymmetric, nearly always in favour of *H. axyridis* (Ware & Majerus, 2008).

Previous studies have determined that PCR cannot distinguish primary from secondary predation (Sheppard *et al*., 2005), or scavenging from predation (Foltan *et al*., 2005; Juen & Traugott, 2005). Detection of secondary predation could occur if a predator ate the target prey for PCR (e.g. *A. bipunctata*), then that predator was itself eaten by the target predator (e.g. *H. axyridis*), leading to inconclusive results. In our system, the likelihood of this occurring was small because *A. bipunctata* was unlikely to be eaten by any species that was itself prey for *H. axyridis* (although possible exceptions are predatory hemipterans). There is a possibility that *H. axyridis* scavenged *Adalia* species rather than predating them, as coccinellids are known to scavenge (i.e. feed on dead remains) (Majerus, 1994). However, it should be noted that laboratory studies have shown that *H. axyridis* larvae actively and aggressively pursue living *Adalia* larvae (Ware & Majerus, 2008).

Our IGP study complements the recent work of Hautier *et al*. (2011), who used a different approach to detect similar levels of IGP by *H. axyridis*, in Belgium. Many coccinellids are protected from predation by the presence of species or genus-specific alkaloids. Thus, Hautier *et al*. (2011) looked for the presence of various exogenous genus-specific alkaloids in the guts of *H. axyridis* larvae, using GC-MS.

The average rate of *Adalia* IGP detection over the 3 years of our study was 12.2%, but with a dramatic, sixfold increase in detection from 2008 (3.7%) to 2010 (22.7%). Hautier *et al*. (2011) reported an IGP rate of *Adalia* species of 17.6% in 2008. This British increase in IGP over the 3 years of our study, to a level similar to that recorded in the Belgian study, is not surprising; the arrival of *H. axyridis* and its establishment as an invasive alien species in Britain lag several years behind that in Belgium (Adriaens *et al*., 2008). In the third year of our study (2010), we found DNA from both *Adalia* species in two *H. axyridis* larvae (4.5%). Six percent of the *H. axyridis* larvae in the Belgian study showed the presence of two alkaloids in their guts – indicating recent IGP on at least two different species.

Intraguild predation by *H. axyridis* has been implicated in the decline of native coccinellids in North America (Gardiner *et al*., 2011). The findings of both our study in England and Hautier *et al*.'s (2011) Belgian study support the hypothesis that IGP is contributing to similar declines in these and other European countries (Roy *et al*., 2012). These two studies raise the question of which approach to detecting IGP is more informative. Neither approach can determine at which stage of the coccinellid prey life cycle IGP occurred: it impossible to know whether the field collected *H. axyridis* larvae that tested positive for *Adalia* DNA had consumed *Adalia* eggs, larvae, pupae, or even adults. Laboratory studies have shown *H. axyridis* predation on eggs (Ware *et al*., 2008), larvae and pupae (Ware & Majerus, 2008). The specificity of the PCR-based DNA detection system is greater than that of GC-MS. We were able to differentiate predation of the two *Adalia* species, *A. bipunctata* and *A. decempunctata*, while the GC-MS detection system could only identify the alkaloid, adaline, common to both species. However, GC-MS can "multiplex", that is, identify multiple products in a single reaction. We needed separate PCR reactions to identify IGP of *A. bipunctata* and *A. decempunctata*. It is possible, although expensive, to develop multiplex PCR systems in which multiple species are simultaneously targeted in a single reaction (e.g. Harper *et al*., 2005 and see Aebi *et al*., 2011 for a review).

An unresolved issue for both PCR based and GC-MS systems of IGP detection is determining with any degree of precision how recently the IGP occurred. Positive detection of, for example, *Adalia* DNA not only raises the question of *when* ingestion of this material occurred, but also of *how much Adalia* tissue was consumed by a *H. axyridis* larva. Laboratory-based controlled feeding trials are useful in giving a broad indication of sensitivity of PCR primers for target DNA, or GC-MS for the alkaloids, but it should be noted that positive detection relates to the amount of material used in the specific feeding trial regime. Thus, considering our PCR detection system, the three larvae that tested positive with the ITS1-AD2 primer pair could be evidencing either recent ingestion of a small amount of *Adalia* tissue or the ingestion of more tissue, but longer ago. There are also a range of factors which may influence digestion rates of DNA and therefore the interpretation of positive results. For example, an insect's metabolic characteristics change both on a diurnal basis and by life stage. The hunger status of a larva may influence digestive enzyme activity. Fluctuations in external temperature, and so enzymatic digestion in the larvae, may also have an effect (Hosseini *et al*., 2008).

Any technique to detect IGP is necessarily going to be a "snapshot in time" – it is not currently possible to precisely state when the predation event occurred. The bigger issue, presented in this article, is that IGP occurred in a wild community and was detected. Furthermore, IGP was detected at all four sites examined in our study, and over its 3 years' duration, the incidence of IGP increased, suggesting an escalating impact of *H. axyridis* predation on *A. bipunctata* and *A. decempunctata*. This work helps to explain an important mechanism behind the invasive success of a species that has spread globally at a remarkable pace (Brown *et al*., 2011b). We intend to devise strategies to look at the wider community effects of the presence of *H. axyridis* on non-coccinellid intraguild and extraguild competitors.

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