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REVIEW

Parasites of *Harmonia axyridis*: current research and perspectives

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Abstract *Harmonia axyridis* (Coleoptera: Coccinellidae) has been introduced widely for biological control of agricultural pests. *Harmonia axyridis* has established in four continents outside of its native range in Asia and it is considered an invasive alien species (IAS). Despite a large body of work on invasion ecology, establishment mechanisms of IAS and their interactions with natural enemies remain open questions. Parasites, defined as multicellular organisms that do not directly kill the host, could potentially play an important role in regulating host populations. This study presents a review of the parasites of *H. axyridis*, discussing their distributions and effects on host populations across the host's native and invasive range. These parasites are: *Hesperomyces virescens* Thaxt. fungi, *Coccipolipus hippodamiae* (McDaniel and Morrill) mites, and *Parasitylenchus bifurcatus* Poinar and Steenberg nematodes.

Keywords *Coccipolipus hippodamiae* · Enemy release hypothesis · *Harmonia axyridis* · *Hesperomyces virescens* · Parasites · *Parasitylenchus bifurcatus*

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Introduction

The harlequin or multicoloured Asian ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) is a striking example of a biological introduction with unintended ecological consequences. *H. axyridis* is native to vast areas in temperate and subtropical (southeastern) Asia, from the Japanese archipelago and Kuril Islands in the east to Western Siberia in the west, with the southernmost localities reported from southern China and Vietnam (Orlova-Bienkowskaja et al. 2015). The most western localities reported by Orlova-Bienkowskaja et al. (2015) lie in longitudes of about 72° E, but some authors (Tyumaseva 1997; Pekin 2007; Khabibullin et al. 2009) report *H. axyridis* as far east as the Ural Mountains (approximate longitude 55° E). It was introduced repeatedly into the USA beginning in 1916 and in Western Europe since the 1980s where it was used as a biocontrol agent against aphids and scale insects.

Multiple introductions of *H. axyridis* to the USA occurred, with distinct founding populations in eastern and western states (Lombaert et al. 2010). Expansion of *H. axyridis* populations across regions in the USA occurred fastest in states with a preponderance of biomes similar to its native habitats in Asia (Koch et al. 2006). *H. axyridis* was first reported as established in the USA in 1988 in southeastern Louisiana and in 1990 in eastern Mississippi (Chapin and Brou 1991). *H. axyridis* has been found in 47 of the 48 contiguous states, leaving Wyoming as the only contiguous state without a record (Roy et al. 2016). In Europe, *H. axyridis* was first recorded in the wild in 1999 in Germany (Brown et al. 2008) and is now considered established in over 30 countries (Aysal and Kivan 2014; Havelka et al. 2015; Kulijer 2016; reviewed in Roy et al. 2016). The first South American (Argentina) record was made in 2001 (Lombaert et al. 2010; Stals 2010). In Africa, *H. axyridis* is known to be most widespread in South Africa where it was first recorded in 2001 (Stals 2010; Roy et al. 2016). Larvae of *H. axyridis* were observed for the first time in New Zealand in 2016 (http://naturewatch.org.nz/observations/3175895), marking the first Oceanian report for this ladybird species. *H. axyridis* is highly competitive with native ladybird species and its strong dispersal capacities allow it to rapidly expand its range into new regions (Roy et al. 2016). Being a globally invasive alien species (IAS) its impacts are considered “immense, insidious, and usually irreversible” (Invasive Species Specialist Group 2000). *H. axyridis* has negative effects not only on non-target insect species (including a decline of native ladybird populations), but also on the food industry and human health (Koch and Galvan 2008). Therefore, it is relevant to determine which natural enemies could have a role in regulating its populations.

Despite a few studies focusing on the suite of natural enemies of *H. axyridis* (Roy and Cottrell 2008; Riddick et al. 2009; Roy et al. 2011; Ceryngier

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et al. 2012; Ceryngier and Twardowska 2013), there is a need for a global overview that includes all reported localities and consideration of biological control potential. Here we present an overview of the parasites of *H. axyridis* and their known global distribution.

Definitions

We restrict usage of the term "parasites" to those organisms living at the expense of a single host, which are multicellular (in contrast to pathogenic microorganisms) and do not directly cause death of the host (in contrast to parasitoids) (Vinson and Iwantsch 1980; van den Bosch et al. 1982; Godfray 1994; Federici 2009). Of the parasites so defined, three species are known to attack *H. axyridis*: the fungus *Hesperomyces virescens* Thaxt., the mite *Coccipolipus hippodamiae* (McDaniel and Morrill), and the nematode *Parasitylenchus bifurcatus* Poinar & Steenberg.

Under this definition, *P. bifurcatus* (Nematoda: Allantonematidae) is a typical member of parasitic nematodes, unlike entomopathogenic nematodes (Heterorhabitidae and Steinernematidae), which form associations with mutualistic bacteria in the genera *Photorhabdus* and *Xenorhabdus* (Grewal et al. 2005). Entomopathogenic nematodes kill their hosts relatively quickly (typically within 24–48 h of infection) aided by their associated bacteria. *P. bifurcatus*, on the other hand, is a typical parasite: it can co-exist with its host for an extended time without killing it. In this respect it is similar to *H. virescens* and *C. hippodamiae*. Nonetheless, entomopathogenic nematodes are considered a subset of parasitic nematodes (Grewal et al. 2005) and are thus also discussed in this manuscript. Here, we review first findings and current distributions of these parasites, explore their potential to regulate *H. axyridis* populations, and outline future research directions.

Hesperomyces virescens (Ascomycota: Laboulbeniales: Laboulbeniaceae)

Hesperomyces virescens is an obligate ectoparasite that has been reported to infect adults of over 30 ladybird species (Coleoptera: Coccinellidae). In addition to the hosts reported by Santamaría et al. (1991) and Ceryngier et al. (2012), the ladybird genera *Azya*, *Epilachna*, and *Halyzia* were recently observed to be hosts of *H. virescens* (Haelewaters and van Wielink 2016; Haelewaters unpublished). *H. virescens* completes its entire life cycle on the integument of a living host where individual yellowish-greenish thalli are formed directly from ascospores (detailed morphology in De Kesel 2011). These thalli can be formed on any part of the body of the insect (Haelewaters et al. 2012) and penetrate the insect cuticle by formation of a haustorium consisting of rhizoids of about 3 µm in diameter (Weir and Beakes 1996). The development from ascospore into mature thallus can take place at temperatures ranging from 10–30 °C. Under laboratory conditions at 25 °C this requires 13–26 days depending on the host species (Cottrell and Riddick 2012). The sticky spores of *H. virescens* have a short life span and are exclusively spread by activities of the host (De Kesel 1995; Cottrell and Riddick 2012).

The first report of *H. virescens* on *H. axyridis* came from North America, dating from July to August 2002, on specimens collected in Ohio (Garcés and Williams 2004). Since then, researchers started looking for this parasitic fungus on *H. axyridis* in the field and in museum collections. Publications of (historical) records followed from countries in five continents (Supporting Material S1). In this paper, new country records of infected *H. axyridis* are reported from Canada, Argentina, Austria, France, and Slovakia.

Experiments and field observations indicate that a number of behavioural and life history traits of *H. axyridis* promotes the spread of the parasite within the populations (Riddick and Schaefer 2005; Nalepa and Weir 2007; De Kesel 2011; Ceryngier and Twardowska 2013). Transmission mostly occurs during sexual contact in the mating/feeding season, indicated by sex-related infection patterns of thalli (Weir and Beakes 1996; Welch et al. 2001; Garcés and Williams 2004; Harwood et al. 2006; Riddick and Schaefer 2005; Ceryngier and Twardowska 2013). In contrast to

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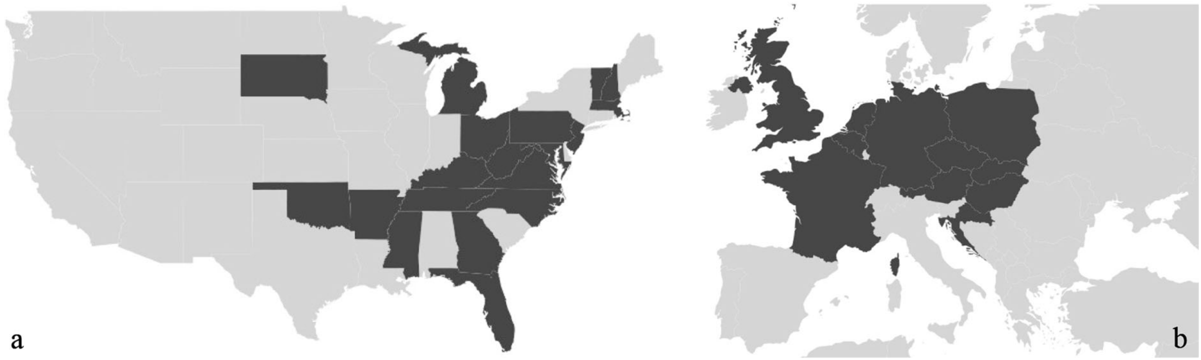

Fig. 1 Detailed distribution of *H. virescens* parasitizing *H. axyridis* in the USA and in Europe. Areas where the association is known are highlighted in *black*. **a**. In the following states of the USA the association has been found: Alabama*, Arkansas, Florida*, Georgia, Kentucky, Maryland*, Massachusetts, Michigan*, Mississippi, New Hampshire*, New Jersey*, North Carolina, Ohio, Oklahoma, Oregon*, Pennsylvania, South Dakota, Tennessee*, Vermont*, Virginia*, and West Virginia. **b**. In Europe, the association *H. axyridis*–*H. virescens* has been reported from Austria*, Belgium, Czech Republic, Croatia, Germany, France*, Hungary, the Netherlands, Poland, Slovakia*, and the UK. In addition to these records, *H. virescens*-infected *H. axyridis* have been recorded in Canada*, Argentina*, Ecuador, South Africa, and China. *new state/country record(s). For details, see Supporting Material S1

other hosts of *H. virescens*, *H. axyridis* combines its multivoltine lifestyle with high promiscuity and the formation of large and very dense overwintering aggregations that randomly boost the transmission of the parasite between cohorts (generations). Autoinfection is caused by grooming, resulting in higher thallus densities on older hosts (Riddick and Schaefer 2005; Haelewaters et al. 2012).

Although current infection rates are high in some southeastern parts of the USA (Fig. 1; Supporting Material S2), *H. virescens* was apparently absent from *H. axyridis* when it first began colonizing North America. A time lag may have occurred between the establishment of *H. axyridis* in the wild (in 1988) and the acquisition of *H. virescens* by this ladybird (in 2002) (Fig. 2; Supporting Material S3). However, insect collections screened for *H. virescens* were not sampled with the fungus in mind and so the prevalence in museum collections reflects natural prevalence, assuming that sampled individuals are representative of the natural populations. In addition to the first reports of *H. virescens* on *H. axyridis* in Ohio, this association was also found in other states in the same year: Virginia (May, $n = 4$), West Virginia (May, $n = 1$), and Tennessee (Jul, $n = 8$) (details in Supporting Material S1). A similar time lag was observed in the Netherlands between 2002 and 2008, when the first record of *H. virescens* on *H. axyridis* was detected during a large-scale study looking for natural enemies (Raak-van den Berg et al. 2014; Supporting Material S3). In a second study from the Netherlands, Coccinellidae have been collected since 1997. Since the first observation of *H. axyridis* (in 2003), almost 8000 specimens have been collected in thirteen consecutive years and screened for *H. virescens* (van Wielink submitted). Again, the first specimen infected with *H. virescens* was observed in 2008 (Haelewaters et al. 2012; van Wielink submitted).

Parasite prevalences of *H. virescens* significantly differ among locations and seasons, but also from one year to another (De Kesel 2011; Haelewaters et al. 2012; Raak-van den Berg et al. 2014; Supporting Material S2). Seasonal variation in prevalence of Laboulbeniales is mainly explained by the emergence of uninfected new generation hosts (Scheloske 1969; Riddick and Cottrell 2010; Haelewaters et al. 2015a), while differences among locations are due to habitat and population density (Scheloske 1969; De Kesel 1996).

In an ongoing study, *H. axyridis* specimens are regularly collected from overwintering aggregations at the Botanic Garden Meise, Belgium. After the first observation of *H. virescens* in the winter of 2007 on a single specimen (from a total $n = 203$), the parasite prevalence of overwintering populations increased rapidly (De Kesel 2011; Supporting Material S2). Inoculation builds up in the dense overwintering aggregations, and thus specimens collected in October–November are usually less infected and also carry a larger fraction of juvenile thalli. Since the

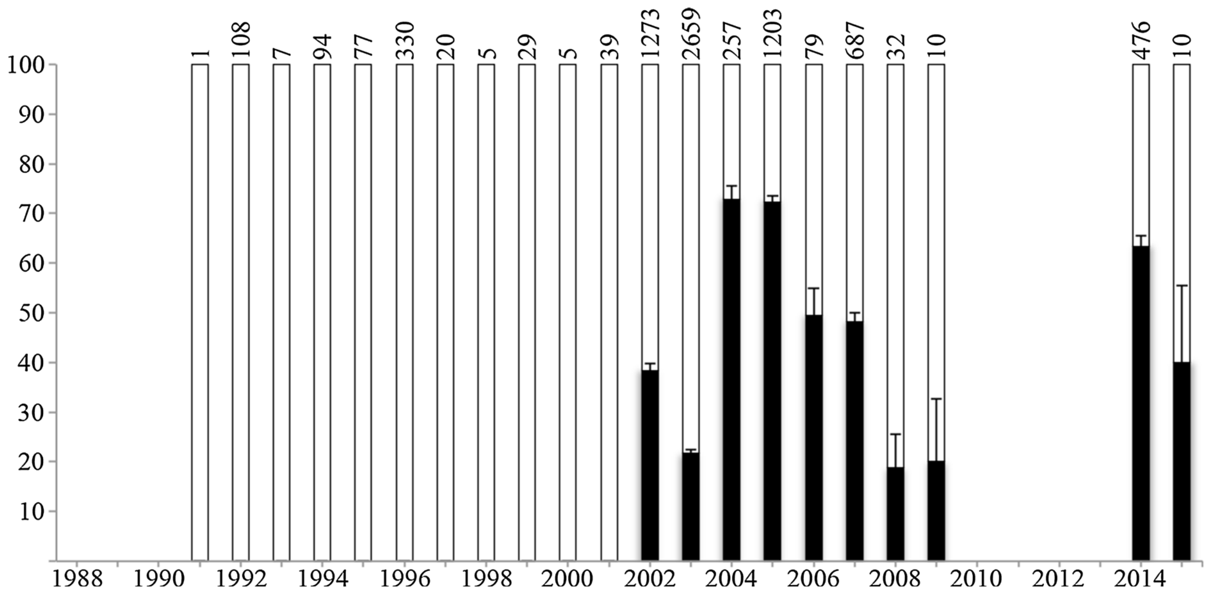

Fig. 2 Prevalence of *H. virescens* on *H. axyridis* adults in North America (Canada, United States) between 1988, when the first established population of *H. axyridis* in the wild was discovered, and 2015 (total $n = 7404$). Individuals from pinned museum collections were screened ($n = 521$) (Haelewaters and Zhao unpublished). In addition, specimens were collected in the field and screened ($n = 486$) (Cottrell and Haelewaters unpublished). Finally, a literature review was done aiming for papers of *H. virescens* on *H. axyridis* with detailed numbers of collected and infected ladybirds ($n = 6397$). *White bars* are uninfected specimens; the *black portions* denote specimens infected with *H. virescens* (in %, with SE). The *horizontal axis* shows the year in which ladybirds have been collected (*bottom*) and number of beetles screened (*top*)

overwintering site is usually dry, water is most probably taken from the host. This is possible since *H. virescens* produces a haustorium, which penetrates through the host cuticle. Despite the high prevalence on *H. axyridis*, no thalli have been observed on other ladybird species overwintering at the same site: *Adalia bipunctata* (L.), *Coccinella septempunctata* L., and *Oenopia conglobata* (L.) (A. De Kesel unpublished). Also Cottrell and Riddick (2012) found reduced interspecific transmission of *H. virescens* (under laboratory conditions) and hence suggested the existence of host-adapted isolates or strains of *H. virescens*.

In the Czech Republic (South Bohemian Region, České Budějovice), prevalence of *H. virescens* remained under 5 % until the autumn of 2014. Of the samples of ladybirds then migrating to overwintering sites, 26 % were infected. Fertilized females were more often infected by *H. virescens* compared to non-fertilized females. Males were more often parasitized than females and bore thalli especially on the ventral side, while female ladybirds bore thalli mostly on the posterior part of the elytra, a result of sexual contact between hosts [sensu Welch et al. (2001), for *A. bipunctata*]. Finally, uninfected males and males with only a small number of thalli had a greater structural size compared to heavily infected males (elytral width × elytral length; 26.0 ± 0.1 mm^2 vs. 25.1 ± 0.3 mm^2, $F_{(1,577)} = 7.79$, $P = 0.0054$; Fiedler and Nedvěd unpublished). This does not mean that infection by Laboulbeniales causes reduced growth, but rather that smaller males may have been more active in mating (thus creating more opportunities for transmission of ascospores) or were more sensitive to infection.

Occurrence in the native range of *H. axyridis*

Studies of museum specimens of Chinese *H. axyridis* ($n = 336$) revealed two specimens dating from the 1930s bearing thalli of *H. virescens* (Haelewaters et al. 2014). Important contributors to the study of Laboulbeniales in the native range of *H. axyridis*, Sugiyama et al. and Terada et al., did not mention *H. virescens*. In Japan, Ceryngier et al. (2012) reported *H. virescens* only from *Coccinula crotchi* (Lewis) and *Coccinula sinensis* (Weise) (subfamily Coccinellinae).

In another study, museum collections in China and Japan of pinned *H. axyridis* from the native range ($n = 1803$; Supporting Material S2) were screened for the presence of Laboulbeniales but did not display infections by *H. virescens* (Zhao unpublished; Supporting Material S4). These collections may include a few specimens of *Harmonia yedoensis* Takizawa which is distinguished from *H. axyridis* only through examination of male genital characteristics that is prohibitive in a large museum survey (Osawa and Ohashi 2008). The single other morphological character for distinction between the two species is the elytral ridge, which is absent in *H. yedoensis* and present in only some *H. axyridis*. These negative results, coupled with only two historical Chinese records of *H. axyridis* infected with *H. virescens*, suggest that parasite prevalence of *H. virescens* on *H. axyridis* in its native range must be (extremely) low.

No other historical or recent records of *H. virescens* on *H. axyridis* are available from the native range, suggesting that uninfected specimens of *H. axyridis* were introduced into North America. *H. virescens* was already reported in North America in the nineteenth century on the host *Chilocorus stigma* (Say) in California, USA (Thaxter 1891). Further records of *H. virescens*, also in Europe, were made long before the introduction of *H. axyridis* (Riddick et al. 2009; Ceryngier et al. 2012; Ceryngier and Twardowska 2013), which suggests one or multiple host shift events.

Negative effects and implications

Given that *H. virescens* is known to infect *H. axyridis* in a rapidly increasing number of countries, locally with very high parasite prevalences (Fig. 1; Supporting Material S2), it may be worth exploring its potential in regulating invasive populations of *H. axyridis*. Moreover, it has the advantage of limited transmission potential to native coccinellids. Indeed, multiple isolates or strains of *H. virescens* may exist that are only virulent to closely related ladybirds or even a single species, a suggestion made based on laboratory transmission experiments (Cottrell and Riddick 2012).

A limited number of studies suggest some negative effects on coccinellid populations: a decrease in mating frequency of females (Nalepa and Weir 2007); hampered sensing ability because of the high number of thalli on head, mouthparts, and antennae (Nalepa and Weir 2007); the inability to use one or more legs by the physical effect of thalli in heavily infected individuals (Haelewaters and Pfliegler unpublished observations); lower survival rates in winter, especially of males (Riddick 2010); and premature mortality (Kamburov et al. 1967; but see Applebaum et al. 1971). Altogether these factors will very unlikely play a significant role in regulating *H. axyridis*. This is mostly due to the fact that the reported negative impacts of *H. virescens* are observed at high thallus densities on older animals that already have copulated and reproduced. We think that the negative impact of *H. virescens* comes too late in the life span of *H. axyridis* to regulate populations.

Future research perspectives

The rapid spread of *H. virescens* (Roy et al. 2011; Haelewaters et al. 2016) and its locally high parasite prevalences on *H. axyridis* (Harwood et al. 2006; De Kesel 2011; Ceryngier and Twardowska 2013; Haelewaters et al. 2016) make it an interesting subject for further research regarding the parasite's effects on the host. Current research is targeted at the interactions between *H. virescens* and the immune system of its host (Murray et al. 2015) and between *H. virescens* and other natural enemies of *H. axyridis* (Haelewaters et al. 2015c).

We assume that after a time lag of two to 16 years (Supporting Material S3), *H. virescens* has shifted from native ladybird species to *H. axyridis*. Considering that transmission and successful development of Laboulbeniales is significantly affected by increased host population density and habitat choice (De Kesel 1993, 1995, 1996), the success of *H. virescens* on *H. axyridis* can probably be attributed to the large overwintering aggregations of *H. axyridis*, in which many encounters occur between infected and uninfected ladybirds. Since habitat choices of ladybird species sometimes overlap for overwintering sites, aggregations of overwintering ladybirds can be heterospecific (Ceryngier 2015) and *H. axyridis* may be involved in such aggregations (Steenberg and Harding 2010). Such situations are known to drastically increase opportunities for inter- and intra-specific transmission, eventually resulting in inter-specific shifts. Also inter-specific copulation attempts by male ladybirds occasionally occur (Majerus 1997). Repeated attempts by abundant male *H. axyridis* to copulate with infected native ladybirds might add to the opportunity for host shift. Hypotheses regarding host shifts can be tested experimentally (e.g. De Kesel 1996) and using a macrogeographical approach with molecular phylogenies, comparing sequences of *H. virescens* from native and invasive hosts. Methods incorporating a molecular phylogenetic component to Laboulbeniales research are currently underway (e.g. Goldmann and Weir 2012; Goldmann et al. 2013; Haelewaters et al. 2015b).

Coccipolipus hippodamiae (Acarina: Podapolipidae)

Coccipolipus hippodamiae is an ectoparasitic mite that was discovered on *Hippodamia convergens* Guérin-Menéville in South Dakota (McDaniel and Morrill 1969). It has been reported on *A. bipunctata* and other ladybird species in the subfamily Coccinellinae in several European countries and Russia, and on *Parexochomus troberti concavus* (Fürsch) and *Exochomus fulvimanus* Weise (subfamily Chilocorinae) in the Democratic Republic of the Congo (Ceryngier et al. 2012). All life stages of the mite live on the underside of the elytra and feed on the haemolymph. Transmission of motile mite larvae occurs during mating of the hosts (Hurst et al. 1995) or, rarely, through close contact in overwintering aggregations of ladybirds (Webberley and Hurst 2002).

Coccipolipus hippodamiae was found for the first time on field-collected *H. axyridis* in 2007 in North America (Mississippi, USA) and in 2009 in Europe (Poland) (Rhule et al. 2010a, b; Riddick 2010). Infection frequencies were 1.8–17.4 and 3.71 %, respectively. The first individuals of *H. axyridis* found with *C. hippodamiae* in the Netherlands were discovered in winter 2009 (Raak-van den Berg et al. 2014), with low parasite prevalences (<3 %) ranging between locations.

Establishment and successful maintenance of *C. hippodamiae* in populations of ladybirds depends on (1) promiscuous behaviour of ladybird hosts to allow for horizontal transmission, and (2) overlapping generations in the field and during overwintering to facilitate inter-generational transmission (Hurst et al. 1995; Pastok et al. 2016). These two requirements are fulfilled in *H. axyridis*, which is highly promiscuous and overwinters in large numbers, allowing for many inter-generational contacts. For these reasons, *H. axyridis* is a good host for *H. virescens* and we believe they also make it a highly suitable host for *C. hippodamiae*.

Coccipolipus hippodamiae has been recorded in Europe since at least the 1980s (Olszak and Suski 1995; Zakharov and Eidel'berg 1997) or even 1960s (as undetermined *Podapolipus* species, Ceryngier and Hodek 1996), before the introduction of *H. axyridis*. This suggests that the mite has recently undergone a host expansion (Raak-van den Berg et al. 2014). *Adalia bipunctata* was found as a common host to *C. hippodamiae* in central, southern, and eastern Europe, but in northern and northwestern populations the mite was entirely absent (Webberley et al. 2006). This was attributed to the fact that in cool climates inter-generational mating of ladybirds, required for transmission of the mite, is rare as the old generation dies before the new generation is reproductively mature (Pastok et al. 2016). *H. axyridis* being a new and potentially suitable host species might expand the northern range of *C. hippodamiae*.

Occurrence in the native range of *H. axyridis*

Thus far, no reports of *C. hippodamiae* on *H. axyridis* are known from its native range. However, parasitic mites of unconfirmed taxonomy were reported by Kuznetsov (1997) to infect several ladybird species (larvae and adults), including *H. axyridis*, in the Russian Far East. These mites were stated as belonging to the family Trombiculidae, which, however, usually infects vertebrates (Zhang 1998; Moniuszko and Mąkol 2014). Much more likely, the mites were representatives of Trombidiidae.

Negative effects and implications

The effects of *C. hippodamiae* have been studied extensively, especially in *A. bipunctata*, and include female sterility and lowered male survivorship during overwintering (Hurst et al. 1995; Webberley and Hurst 2002; Webberley et al. 2004). As in *H. axyridis*, infection with *C. hippodamiae* causes female infertility from 19 days post-infection onwards (Rhule et al. 2010b). Under simulated winter conditions (8 °C and 58–60 % RH), Riddick (2010) observed no mortality of *C. hippodamiae*-

infected *H. axyridis* adults (all females) and concluded that *C. hippodamiae* has no influence on winter survival of females. However, *H. axyridis* infected with both *H. virescens* and *C. hippodamiae* experienced lower survival under the same conditions. Rhule et al.'s (2010b) and Riddick's (2010) findings call for further work in developing this natural enemy as a potential biological control agent. However, it will be important to consider the potential deleterious non-target effects.

Future research perspectives

Our present knowledge on the relationships between *H. axyridis* and *C. hippodamiae* is highly incomplete with preliminary data suggesting strong negative effect of the mites on ladybird fertility and less pronounced effect on their survivorship. Further laboratory and field experiments quantifying the negative impacts and rates of transmission under various environmental conditions are required.

Geographical distribution of the *H. axyridis*–*C. hippodamiae* association is also poorly investigated. To fill the gaps, a large-scale search for parasitic mites should be conducted in the native and invasive range of *H. axyridis*. *C. hippodamiae* has been reported from other ladybird hosts from North and Central America, Africa, Europe, and the western edge of Asia (Georgia) (Ceryngier et al. 2012), confirming a broad global distribution. Moreover, some other species of *Coccipolipus* can possibly adapt to exploit *H. axyridis*. Of fifteen known species in this genus, most have exclusively been found either on the members of Epilachninae (seven species) or Chilocorinae (three species), but four species other than *C. hippodamiae* (*C. cooremani* Husband, *C. macfarlanei* Husband, *C. micraspisi* Husband, and *C. synonychae* Ramaraju & Poorani) are also known to parasitize ladybirds in the subfamily Coccinellinae (Ceryngier et al. 2012; Ramaraju and Poorani 2012).

On the other hand, it seems possible that the *Coccipolipus* mites will remain only occasional parasites of *H. axyridis*. While *H. virescens* has recently been found associated with *H. axyridis* in many new localities, this is not the case with *C. hippodamiae*. Since its discovery several years ago in one North American and two European *H. axyridis* populations, no further records of this association have been reported.

***Parasitylenchus bifurcatus* (Nematoda: Allantonematidae)**

In contrast to the ectoparasitic laboulbenialean fungi and podapolipid mites, nematodes infecting Coccinellidae live in the body cavity of their hosts. Associations between ladybirds and nematodes have been observed or suggested for more than 100 years, e.g. at least since the report of Linstow (1899) in which *Mermis nigrescens* Dujardin (Mermithida: Mermithidae) was suggested as a parasite of *C. septempunctata* (Poinar 1979). However, relatively few reports of parasitism in nature have ensued. Moreover, when restricting the association to *H. axyridis*, only members of a single nematode family, Allantonematidae, have been documented as a natural parasite.

Parasitism of *H. axyridis* by *Parasitylenchus* sp. (Tylenchida: Allantonematidae) was first reported by Harding et al. (2011). The infected insects were collected in Denmark. The nematode was later described as a new species, *P. bifurcatus* (Poinar and Steenberg 2012). The new species was distinguished from the previously described *Parasitylenchus coccinellinae* Iperti & van Waerebeke, which had been discovered parasitizing other species with multivoltine life cycles [*Propylea quatuordecimpunctata* (L.), *O. conglobata, A. bipunctata, Hippodamia variegata* (Goeze)] in France (Iperti and van Waerebeke 1968). Subsequently, *P. bifurcatus* was also found parasitizing *H. axyridis* in the Netherlands since 2008, the Czech Republic in 2012, and Poland in 2013 (Supporting Material S1). Unidentified nematodes in the family Allantonematidae were isolated from *H. axyridis* in Germany (Herz and Kleespies 2012) and Minnesota, USA (Roy et al. 2011). For parasite prevalences, see Supporting Material S2.

Occurrence in the native range of *H. axyridis*

According to Kuznetsov (1997), infections of *H. axyridis* in its native range were documented by unidentified nematodes of unconfirmed taxonomy. These infections were reported in the Primorsky Territory in the Russian Far East on the following ladybird species: *Aiolocaria hexaspilota* Hope, *C. septempunctata*, *H. axyridis*, *Hippodamia tredecimpunctata* (L.) (subfamily Coccinellinae), *Chilocorus inornatus* Weise and *C. rubidus* Hope (subfamily

Chilocorinae). The parasite prevalence was always below 2 %.

Negative effects and implications

Nematodes may offer some level of natural control against *H. axyridis* in its invasive range. Based on the observed rates of infection and potential mortality or reduction in fitness, Poinar and Steenberg (2012) suggested that *P. bifurcatus* could be a significant biocontrol agent of *H. axyridis*. In Denmark, parasite prevalences up to 35 % were observed (in 2010), and in the Czech Republic even as high as 47 % (in 2014).

Parasitylenchus bifurcatus is apparently capable of causing significant harm to its host and infection may occur at moderate rates. It was observed that *P. bifurcatus* caused depletion of fat body in *H. axyridis* as well as partial or complete atrophy of the insect's reproductive organs (Poinar and Steenberg 2012). However, Fiedler and Nedvěd (unpublished) found no difference in body mass between infected and uninfected *H. axyridis* in the Czech Republic ($n = 49$). Infections occurred throughout the year with parasite prevalence reaching up to 35 % (Harding et al. 2011; Poinar and Steenberg 2012), and in females even up to 47 % (Fiedler and Nedvěd unpublished). Regarding the mode of transmission, Poinar and Steenberg (2012) suggested that the infected females pass from one adult host to another when the beetles are aggregated (e.g. during overwintering).

In addition to parasitism by nematodes in nature, Shapiro-Ilan and Cottrell (2005) measured the innate susceptibility of *H. axyridis* to infection by entomopathogenic nematodes (Rhabditida: Heterorhabditidae and Steinernematidae) under laboratory conditions. Entomopathogenic nematodes are commonly used as biological control agents for a wide variety of economically important insect pests (Grewal et al. 2005). Pathogenicity, virulence, and reproductive capacity of *Heterorhabditis bacteriophora* Poinar and *Steinernema carpocapsae* (Weiser), isolated from geographic areas overlapping with those of the studied ladybirds, were compared among two native [*Coleomegilla maculata* and *Olla v-nigrum*] and two established alien (*H. axyridis* and *C. septempunctata*) ladybirds. Another insect, *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae), that is known to be susceptible to the nematode species, was also included for host comparisons. The ladybird species were less susceptible than the target pest (*A. ipsilon*). Also, it was observed that, compared to the target pest, the ladybird species were less likely to come in contact with entomopathogenic nematodes following soil applications for biocontrol purposes. Therefore, field applications of entomopathogenic nematodes would likely have significantly less impact on ladybird populations than on a susceptible target pest.

Additionally, alien ladybirds were found to be less susceptible to entomopathogenic nematode infection than native species (reproductive capacity of nematodes in *H. axyridis* was also lower than in the native species). Thus, the hypothesis that low susceptibility may have contributed to competitive establishment due to enemy release was supported in the study (Shapiro-Ilan and Cottrell 2005). Naturally occuring infections of *H. axyridis* with nematodes in its invaded range, however, may be good starting points for biocontrol research (Harding et al. 2011).

Future research perspectives

As indicated above, *P. bifurcatus* may have potential as a biocontrol agent of *H. axyridis*. Additional research is needed to explore this possibility or the potential of other nematodes as regulating factors of *H. axyridis* populations. The aggregative behaviour of *H. axyridis* during overwintering periods may offer a significant opportunity to utilize *P. bifurcatus* in an effective manner. However, a substantial drawback would be generating sufficient quantities of the nematode. In vivo production in the natural host will be costly.

Application of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) for the regulation of *H. axyridis* would be far more cost-effective given that these nematodes are already produced and sold commercially as biocontrol agents (Grewal et al. 2005). However, the entomopathogenic nematodes that have been tested thus far are not highly virulent to *H. axyridis* (Shapiro-Ilan and Cottrell 2005). Conceivably, other species or strains of entomopathogenic nematodes that have not been tested, particularly those found in the native range of *H. axyridis*, may be more virulent. The risks and benefits of potential releases would have to be weighed and based on pertinent data such as non-target studies and the biological histories of the exotic entomopathogenic nematodes and local ecosystems. Thus far, introductions of entomopathogenic nematodes have proved benign to non-target species and resulted in efficient biocontrol (Ehlers 2005).

One barrier to the use of entomopathogenic nematodes for *H. axyridis* control is that high levels of RH are required for nematode survival and activity, and thus target areas in which *H. axyridis* resides may not be conducive. On the other hand, various gels or other formulations can protect the nematodes from desiccation and therefore may facilitate successful biocontrol (Shapiro-Ilan and Dolinski 2015; Shapiro-Ilan et al. 2016).

As entomopathogenic nematodes have been used in integrated pest management programs for decades, a growing body of literature is available about abiotic factors that influence survival and infection success. For *P. bifurcatus*, this is not the case because it is currently not at all in use as a biological control agent. However, it seems clear that the abiotic requirements are not as restricted as those associated with entomopathogenic nematodes: *P. bifurcatus* is found naturally infecting *H. axyridis* under natural conditions such as above-ground on various plant species (Harding et al. 2011; Poinar and Steenberg 2012).

Support for the enemy release hypothesis?

The enemy release hypothesis (ERH), or enemy escape hypothesis, provides a framework in which interactions between IAS and natural enemies can be explored (Jeffries and Lawton 1984; Colautti et al. 2004; Roy and Lawson Handley 2012). ERH has been widely discussed but, in the context of many invasions, there is a lack of robust evidence to underpin the theory. ERH predicts that an alien species invading new geographic areas will experience reduced pressure from natural enemies, compared to native (or alien non-invasive) species. This, in turn, will lead to population increases of the alien species in its invasive range. However, this escape-from-enemies effect could be lost with increased residence time and spread in the introduced range, as IAS acquire new enemies (=New Associations Hypothesis, Hokkanen and Pimentel 1989; Siemann et al. 2006; Schultheis et al. 2015). Hence, invasions are dynamic and it is important to reinforce that in systems that illustrate ERH, the prevalence of natural enemies for IAS is lower only at early stages of the invasion process.

In assessing whether or not *H. axyridis* has benefited from enemy release, we should consider presence/absence and the effects of natural enemies on native and invasive populations of *H. axyridis* on the one hand ("biogeographical studies"), and invasive populations of *H. axyridis* and co-occurring native species ("community studies") on the other hand (Colautti et al. 2004). Another feature for consideration is the acquisition of new enemies by *H. axyridis* in its invasive range (which implies host shifting by these native natural enemies).

In the literature available for parasites of *H. axyridis*, support for ERH is available from a community approach, but lacking from a biogeography approach. Shapiro-Ilan and Cottrell's (2005) community study found that *H. axyridis* was less susceptible to entomopathogenic nematodes compared to the native ladybird species, providing direct support for ERH. Other studies (Rhule et al. 2010b; Riddick 2010) provide data about the negative effects of *C. hippodamiae* and/or *H. virescens* on *H. axyridis* (female sterility, mortality) but do not compare invasive *H. axyridis* populations with native populations or native ladybirds in the invasive range. Further support for ERH can be inferred from the higher prevalence of *C. hippodamiae* in native *A. bipunctata* than in invasive *H. axyridis* in Europe (Rhule et al. 2010b; Ryder et al. 2014).

Some reports are also available on *H. axyridis* acquiring new natural enemies (sensu Hokkanen and Pimentel 1989) in North America and Europe. In both regions of the invasive range, a lag time was observed between the establishment of *H. axyridis* in the wild and the first report of parasites on *H. axyridis*. One study came with support from the Netherlands (Raak-van den Berg et al. 2014), where, since 2002, *H. axyridis* populations were monitored for natural enemies and only in 2008 and 2009 the first observations were made of *H. virescens*, *C. hippodamiae*, and *P. bifurcatus*. A similar study focused on *H. virescens* only in North America and found, between 1988 and 2009, infections only starting in 2002 (and then in every year since) (Haelewaters and Zhao unpublished; Fig. 2). In Supporting Material S3 we summarise first records of the three parasites on *H. axyridis* in different countries in North and South America, Europe, and Africa. Comparing the dates of these first records with the years in which *H. axyridis* was considered established in the respective countries provides additional support for the acquisition of natural enemies after a time lag. This time lag ranges between two years (Denmark) and 16 years (Canada).

Further work is necessary to verify enemy function and distributions across the whole *H. axyridis* range. However, ERH is likely to play a contributing role in the success of *H. axyridis* invasions in addition to its suite of life-history traits conferring advantages in invasion (habitat, diet plasticity, cold tolerance, phenotypic plasticity, reproductive success; Roy et al. 2016).

Co-infections

Within natural systems, hosts are likely to be exposed to, and potentially exploited by, more than one natural enemy interacting with each other directly and indirectly, as well as with the host (Furlong and Pell 2005). These interactions may result in mixed or concurrent infections that can determine the severity of natural enemy impacts at the individual level (such as changes to host fitness or mortality) and thereby influence changes in host population regulation. The role of complexes of natural enemies is recognised in improving insect pest suppression (e.g. Jabbour et al. 2011) but our understanding of multiple natural enemy interactions and the impact on natural populations of invertebrates including ladybirds is very limited. In concluding that natural enemies have only limited potential to control populations of *H. axyridis*, we do so with the assumption that they are acting in isolation.

There is evidence however, albeit from a limited number of studies, that co-infections of natural enemies occur in *H. axyridis*. During a field survey in the Netherlands to specifically identify natural enemies on *H. axyridis* (Raak-van den Berg et al. 2014), two ladybirds were found with both *C. hippodamiae* and *H. virescens* whilst 23 individuals were infected with both *H. virescens* and *P. bifurcatus* ($n = 1429$). Riddick (2010) identified *H. axyridis* co-infected with *C. hippodamiae* mites and *H. virescens*. In this case, the co-infection led to a lower adult winter survival compared to infection with *C. hippodamiae* mites or *H. virescens* alone. It is suggested that this is potentially more significant for males than females, although the number of individuals studied was limited and more data are required to substantiate this result. Similarly, Raak-van den Berg et al. (2014) demonstrated a positive association between nematodes and *H. virescens* at some sites sampled that correlated with a reduced number of live beetles. It was hypothesized that co-infection with nematodes and *H. virescens* might result in lower survival rates, but to date no experimental data has been generated in support of this hypothesis.

Another factor that may influence the survival outcome of co-infection is body mass of the host ladybird. For example, it is well recorded that invertebrates become increasingly resistant to infection as a function of age that may be related to body mass (Groove and Hoover 2007). In a study from the Czech Republic, out of 49 overwintering females in November 2014, 12 were found infected with *H. virescens*, 23 with *P. bifurcatus*, and seven with both parasites (Fiedler and Nedvěd unpublished). Co-infected females had higher body mass ($F_{(1,47)} = 4.81$, $P = 0.03$) and they had higher carotenoid contents in elytra ($F_{(1,47)} = 11.08$, $P = 0.0017$), which indicated that these females were older than uninfected or single infected ones. The age difference may account for changes in body mass: older females had time to accumulate reserves and also to accumulate parasites.

Although there have been many reports of Laboulbeniales infections having a negligible impact on their hosts (Whisler 1968; Scheloske 1969; Riddick et al. 2009), Konrad et al. (2015) report a case where Laboulbeniales infection may be advantageous for the host. The authors show a case of decreased susceptibility to *Metarhizium brunneum* Petch experimental infection in *Lasius neglectus* van Loon, Boomsma et Andrásfalvy ants hosting *Laboulbenia formicarum* Thaxt. (Konrad et al. 2015). This was hypothesised to be due to increased competitive interactions between the fungi or alternations in behaviour and physiological host defences in those individuals carrying *Laboulbenia* that provided protection against subsequent challenge with *M. brunneum*. Such experiments have not been conducted on ladybirds. Given that Coccinellidae are often infected with pathogenic isolates of the generalist fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. (Roy and Cottrell 2008), it will be interesting to determine whether or not carrying *Laboulbeniales* fungi conferred additional protection against infection for *H. axyridis* challenged by *B. bassiana* (Haelewaters et al. 2015c).

There are fundamental gaps in our understanding of the ecology of entomopathogen co-infections, which, together with a lack of empirical data, makes it difficult to predict impacts on insect populations (Hesketh et al. 2010). The data to date suggest that multiple infections at one time are rather rare in *H. axyridis*. However, studies do not regularly include multiple pathogen groups and often are at a single sampling time point rather than across life-stages, making accurate assessment of prevalence challenging. Concurrent infections during the lifetime of individuals may be more common than are currently recorded and expose hosts to multiple pathogen pressures, which have the potential to impact on populations through multiplicative effects.

Conclusions and future directions

The interplay between parasites and IAS represents a fascinating and important component of invasion ecology. It is widely recognised that consideration of parasites is neglected when considering the impacts of an IAS despite the potential importance of such interactions in the outcome of invasion (Dunn 2009). Here we have provided an overview of current understanding of the natural enemies of *H. axyridis*. Although there have been considerable advances in recent years, there is still a need for large-scale systematic studies to reveal the strength and importance of host-parasite interactions in different biogeographic contexts. Fundamentally, we still have limited understanding of the regulatory effects of natural enemies on ladybird populations. There are opportunities to collaboratively use *H. axyridis* as a study system for assessing natural enemy interactions on a global scale. A first step would be to agree upon essential variables for the systematic assessment of such interactions. Network approaches (Roy and Lawson Handley 2012) could prove illuminating and advances in molecular tools will enable analyses on scales previously considered inconceivable.

Further studies of co-infections and co-occurences of parasites (and pathogens) on *H. axyridis* and their effects on the hosts' fitness (e.g. reproductive success, overwintering ability, susceptibility to chemical agents, etc.) are essential to identify opportunities for natural enemy suppression of *H. axyridis*. Potentially, increased population regulation may be achieved if synergism between natural enemies in reducing host survival/fitness can be exploited. It will be important to consider how such interactions vary under different biotic and abiotic conditions and at different spatial and temporal scales. Such opportunities will be enhanced if a suite of natural enemies can be identified from which combinations can be further tested.

Lastly, there is potential to design citizen science initiatives aimed at documenting natural enemy interactions and to provide long-term monitoring data in order to understand how parasite prevalences on *H. axyridis* change over time. However, we emphasize the complexity of host-parasite systems and adequate resources and support will be required to ensure the success of citizen science. Without additional support, the pool of volunteers for recording ladybird parasites might be substantially smaller than the pool willing to record ladybirds in general. Indeed a UK initiative (http://www.bbc.co.uk/breathingplaces/ladybird-parasites/) to engage people in recording ladybird parasites had very low uptake although the resulting data from the few contributors was of high quality (Comont et al. 2014; Roy and Brown 2015). Also in the USA, an effort is being made to incorporate citizen science submission of *H. virescens* sightings on ladybirds, through the Lost Ladybug Project (http://www.lostladybug.org/laboulbeniales-1124.php). Finally, and interestingly, new reports suggesting a further northward (Canada) and southward (Argentina) spread of the *H.axyridis–H. virescens* association in the western hemisphere were discovered on digital photo- and biological observation-sharing websites Flickr and iNaturalist. Monitoring with a global perspective, with the aid of citizen science efforts, can build an integrated understanding of *H. axyridis* both for management of biological control, and as a model for the study of IAS.

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