frontiers in Ecology and Evolution

MINI REVIEW
published: 31 January 2022
doi: 10.3389/fevo.2022.756972

Double Infections of the Invasive Ladybird *Harmonia axyridis*

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The dramatic spread of invasive alien species over the past century is considered to be an important threat to ecosystems worldwide. The harlequin ladybird, *Harmonia axyridis*, from eastern Asia, is considered to be one of the most invasive species. Originally introduced across the world as a biological control agent against crop pests owing to its voracious appetite and hardiness, those same qualities have made *H. axyridis* an invader that is difficult to eradicate. *Harmonia axyridis* has proven resilient against a variety of pathogens that have negative effects on other coccinellids. However, little research has examined the effects of simultaneous infections of multiple natural enemies on *H. axyridis*. Here we present the available information on such double infections on *H. axyridis*, and discuss further research directions in this area.

Keywords: biocontrol, ecology, entomology, integrative pest management, invasion biology, parasitology

OPEN ACCESS

Edited by:
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Reviewed by:
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Ecdysis Foundation, United States

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Specialty section:
This article was submitted to Population, Community, and Ecosystem Dynamics, a section of the journal Frontiers in Ecology and Evolution

Received: 11 August 2021
Accepted: 10 January 2022
Published: 31 January 2022

Citation:
de Groot MD and Haelewaters D (2022) Double Infections of the Invasive Ladybird Harmonia axyridis. Front. Ecol. Evol. 10:756972. doi: 10.3389/fevo.2022.756972

INTRODUCTION

The introduction and dramatic spread of non-native species is seen as a significant environmental disturbance threatening ecosystems around the world (Mooney and Cleland, 2001; Simberloff and Gibbons, 2004). Human activity enables species to reach and establish themselves in regions outside their native range, known as biological invasions (Vitousek et al., 1996; Mack et al., 2000). Environmentally problematic species are increasing globally, even in areas that are traditionally seen as sources rather than targets of alien species, such as Europe (Hulme et al., 2009). Global trade and human movement exacerbate the rate of alien invaders establishing themselves in new environments, and this method of distribution is projected to accelerate in the future (Levine and D'Antonio, 2003; Pfliegler et al., 2018). Some invasive species have considerable negative environmental and socio-economic effects, leading to mounting efforts to mitigate the damage (Vilà et al., 2010).

One of the posited explanations for the success of invaders in new environments is the enemy release hypothesis (Keane and Crawley, 2002; Colautti et al., 2004; Liu and Stiling, 2006; Roy et al., 2011a). The enemy release hypothesis proposes that population growth of invasive species results from release from their natural enemies that did not co-evolve in the new geographic location with them. This is beneficial for the invasive species on two fronts: first, they are not regulated by specialists, and second, their direct competitors of similar ecological guilds do have specialist enemies. Over time, population dynamics will be affected by evolutionary processes and the amount of natural enemies of the invasive species can increase (Brändle et al., 2008; Schilthuizen et al., 2016; Haelewaters et al., 2017), but not necessarily in time before the ecosystem is harmed (Geschke, 2019).

The harlequin ladybird, *Harmonia axyridis* (Pallas) (Arthropoda: Coleoptera, Coccinellidae), is a ladybird native to eastern Asia. Due to its resilience and voracious appetite, it was deemed very useful as a biological control agent against aphids, coccids, and other pests, and has been widely used in horticulture (Koch, 2003). *Harmonia axyridis* was introduced to protect crops in North America and later as an augmentative biocontrol in Europe, but it has now spread at an explosive rate to every continent except Australia and Antarctica over the past 30 years (Brown et al., 2011; Roy et al., 2016; Camacho-Cervantes et al., 2017; Hiller and Haelewaters, 2019). The same qualities that make *H. axyridis* a successful biocontrol agent also make it an effective intraguild predator. Whereas *H. axyridis* in its native range reached an equilibrium with its co-evolved guild of predators, in its exotic range it is in direct competition with populations of native predators, as the enemy release hypothesis would predict (Pell et al., 2008).

Harmonia axyridis has a negative effect not only on native insects, such as causing local ladybird populations to decline (Mizell, 2007; Roy et al., 2012), but also on food production and human health (Koch, 2003; Pickering et al., 2004; Koch and Galvan, 2008). It has been described as one of the "worst" invasive alien species of Europe (Nentwig et al., 2018). The International Union for Conservation of Nature (IUCN) established a Ladybird Specialist Group to identify species that are threatened and to develop conservation management strategies to counteract factors leading to high extinction risk. New methods of *H. axyridis* control must be assessed for the IUCN and similar groups to succeed in their plans to protect threatened species. In assessing how invasive species like *H. axyridis* can be controlled, it is important to determine the natural enemies of *H. axyridis*, how they spread, and which role they may have in regulating invasive populations of *H. axyridis*.

The search for natural enemies of *H. axyridis* has identified a variety of pathogens, parasites, and parasitoids affecting the ladybird, including bacteria, protozoans, fungi, nematodes, mites, wasps, and flies (Ceryngier et al., 2012, 2018; Haelewaters et al., 2017). Most of these natural enemies are generalists for coccinellid species or other insects. Part of *H. axyridis*' competitive success may be explained by its high resistance to such generalist enemies; *H. axyridis* has a relatively greater efficiency of its immune system, giving it robust and flexible defenses that surpass other competing ladybirds in the same region (Roy et al., 2008, 2011b; Vilcinskas et al., 2013; Gegner et al., 2018; Fincham et al., 2019). The immune system of *H. axyridis* is two-layered—it combines constitutive chemical defenses effective against a variety of bacteria as well as a wide range of antimicrobial peptides that are a result of multiple gene duplication events after speciation (Vilcinskas et al., 2013). Additionally, *H. axyridis* possesses strong alkaloid chemical defenses against predators and pathogens, which give it a foul smell and taste. These *H. axyridis*-specific alkaloids have been found to be more toxic compared to other coccinellids (Röhrich et al., 2011; Sloggett et al., 2011).

While local natural enemies of ladybirds have started using *H. axyridis* as a new host in places where it is invasive (Raak-van den Berg et al., 2014; Knapp et al., 2019), some invasive populations of *H. axyridis* may be only rarely infected (Dudek et al., 2017; Romero et al., 2020). As a result, these enemies of *H. axyridis* only have limited biocontrol potential for controlling invasive populations on their own, and some may actually have more adverse effects on locally native species than on *H. axyridis* (Riddick, 2010; Haelewaters et al., 2017; Ceryngier et al., 2018). However, with this many potential natural enemies affecting *H. axyridis* and the species being so widespread, there might be combinations of pathogens, parasites, or parasitoids that have an adverse effect on the invasive populations. Here, we inventorize previously described double infections of *H. axyridis* and their effects, where known (**Table 1**).

DOUBLE INFECTIONS

Natural enemies impact their hosts' fitness in a variety of ways, such as increasing mortality, decreasing fecundity, and increasing susceptibility to parasites, pathogens, predators, or chemical agents. Insects are susceptible to infection by many different enemies, and simultaneous infections are anticipated to be common in the wild. In attempting to control invasive species, the additive effects of multiple infections can be a way to increase the efficiency of integrated pest management (IPM) programmes (Jabbour et al., 2011; Zindel et al., 2011). However, interactions between infecting species are difficult to predict, and can have conflicting results (**Figure 1**). For example, use of two species of natural fungal pathogens to combat the leaf-cutter ant *Acromyrmex lundii* (Guérin-Méneville) (Arthropoda: Hymenoptera, Formicidae), an agricultural pest in the Neotropics, caused the two species of fungi to suppress each other rather than the pest (Folgarait et al., 2011). In a another example, when the invasive garden ant *Lasius neglectus* Van Loon, Boomsma & Andrasfalvy (Formicidae) was exposed to the mortal entomopathogen *Metarhizium brunneum* Petch (Ascomycota: Hypocreales, Calvicipitaceae) after infection by *Laboulbenia formicarum* Thaxt. (Ascomycota: Laboulbeniales, Laboulbeniaceae), the upregulated immunological and behavioral responses caused fewer fatalities from the secondary infection than either fungus on its own (Konrad et al., 2015). Such complex interactions therefore warrant exhaustive review and experimentation, both by surveys in the wild and mortality experiments in laboratory settings, especially for species with hardy defense systems, such as *H. axyridis.*

The endosymbiotic bacterium *Spiroplasma* (Tenericutes: Entomoplasmatales, Spiroplasmataceae) infects the gut of hemolymph of a large variety of insects, with effects ranging from mutualistic to parasitic (Hackett and Clark, 1989). In a subset of insects including ladybirds, *Spiroplasma* causes so-called male-killing by destroying eggs fertilized by Y-bearing sperm, causing extremely female-biased offspring (Zakharov et al., 1999; Harumoto and Lemaitre, 2018). In *H. axyridis*, *Spiroplasma* also reduces body size, embryo survival, and adult lifespan in infected females (Majerus, 2002). Infection by *Spiroplasma* only seems to occur in native populations of *H. axyridis*; thus far infections have not been reported in populations in areas where they are invasive (Goryacheva et al., 2017). A survey of *Spiroplasma* strains in *H. axyridis* from Japan and eastern Russia—both in its native range—revealed the majority of beetles that were infected by *Spiroplasma* to be infected by multiple strains (88%) (Goryacheva et al., 2018). However, the exact

TABLE 1 | Known simultaneous infections by multiple natural enemies on *Harmonia axyridis* and their effect on the host.

Species 1	Species 2	Field or lab	Locality	Effect of co-infection	Reference(s)
Spiroplasma strain 1	*Spiroplasma* strain 2	Field	Japan, Russia	N/A	Goryacheva et al., 2018
Hesperomyces virescens	*Parasitylenchus bifurcatus*	Field	Germany, Netherlands, Caucasus	N/A	Herz and Kleespies, 2012; Raak-van den Berg et al., 2014; Orlova-Bienkowskaja et al., 2018
Hesperomyces virescens	*Coccipolipus hippodamiae*	Field, Lab	Austria, United States	Increased mortality	Christian, 2002; Riddick, 2010
Hesperomyces virescens	*Beauvaria bassiana*	Lab	United Kingdom, United States	No increased mortality	Berry, 2017; Haelewaters et al., 2020
Hesperomyces virescens	*Metarhizium brunneum*	Lab	United States	No increased mortality	Haelewaters et al., 2020

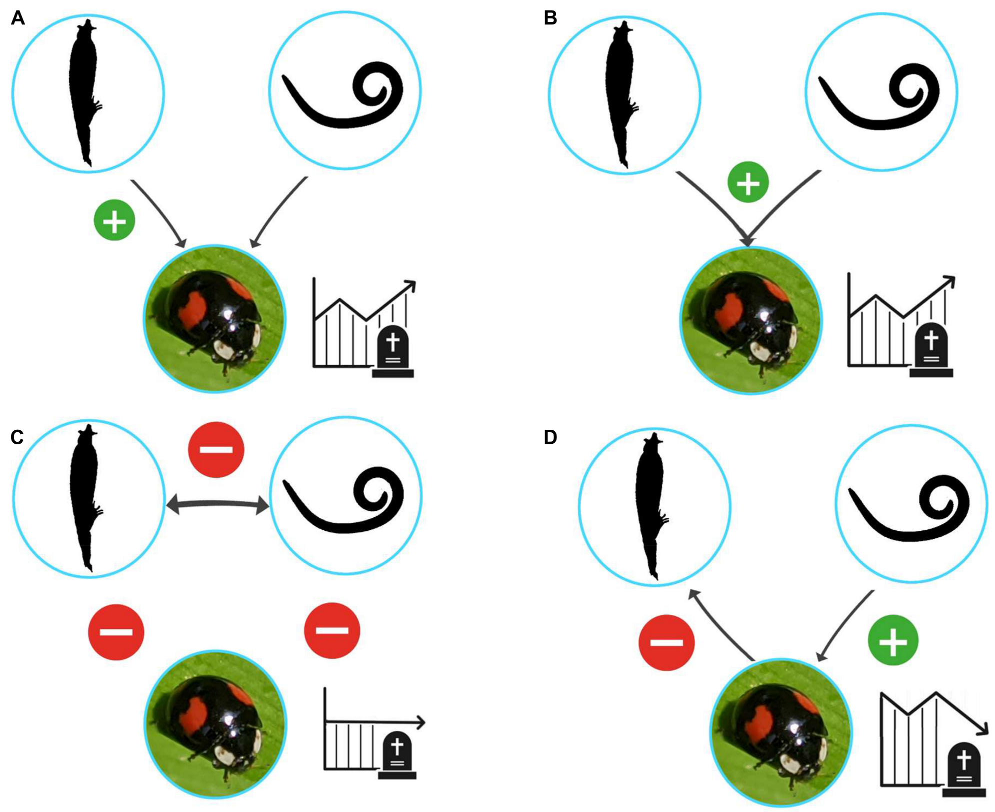

FIGURE 1 | Conceptual overview of potential effects of co-infection by two natural enemies on the fitness of a given host. **(A)** The co-infection causes no additional decrease in fitness. **(B)** There is an additive effect of the second enemy, causing a stronger decrease in fitness upon co-infection. **(C)** The infecting species neutralize each other, causing no increase or decrease in fitness. **(D)** One natural enemy causes a prophylactic effect in the host, preventing a secondary infection from taking hold, increasing the host's fitness. Photo: *Harmonia axyridis* (iNaturalist #53583172).

biological consequences of multiple infections as opposed to a single infection remain unclear.

The fungus *Hesperomyces virescens* Thaxt. (Ascomycota: Laboulbeniales, Laboulbeniaceae) is an obligate ectoparasite infecting adult ladybirds of over thirty species (Haelewaters et al., 2017). Recent integrative taxonomic analyses have revealed that *H. virescens* is in fact made up of many different species, each specifically adapted to its individual host (Haelewaters et al., 2018; Haelewaters and De Kesel, 2020; Crous et al., 2021). *H. virescens* completes its entire lifecycle on its living host; sexual spores divide mitotically to produce yellowish, multicellular, three-dimensional structures called thalli on the outside of any part of the host's body. These thalli penetrate the host's cuticle via a rhizoidal haustorium (De Kesel, 2011; Haelewaters et al., 2017). *Hesperomyces virescens* has been found to co-infect *H. axyridis* hosts alongside the nematode *Parasitylenchus bifurcatus* Poinar & Steenberg (Nematoda: Tylenchida, Allantonematidae) in Germany (Herz and Kleespies, 2012), The Netherlands (Raak-van den Berg et al., 2014), and the Caucasus (Orlova-Bienkowskaja et al., 2018). *Parasitylenchus bifurcatus* is an obligate endoparasite specific to *H. axyridis*; these nematodes live, mate, and proliferate within the host. The method of transmission from host to host is unknown (Poinar and Steenberg, 2012). It was speculated from observations in the field that simultaneous infection by

H. virescens and P. bifurcatus reduced survival rates of *H. axyridis* (Raak-van den Berg et al., 2014).

A different natural enemy to co-infect *H. axyridis* alongside *H. virescens* is the ectoparasitic mite *Coccipolipus hippodamiae* (McDaniel & Moril) (Arthropoda: Acarina, Podapolipidae). Simultaneous infections by these two natural enemies have thus far been observed in Austria (Christian, 2001, 2002) and the United States (Riddick, 2010). *Coccipolipus hippodamiae* infection causes decreased fecundity, decreased egg viability, and increased mortality in a variety of coccinellid species (Webberley et al., 2004), but the exact biological mechanisms are still unknown. Transmission from one host to the next occurs similarly to *H. virescens*: through bodily contact during mating or overwintering (Knell and Webberley, 2004). Mortality from *C. hippodamiae* infection is especially high in overwintering males (Webberley et al., 2006). In a simulated winter experiment, *H. axyridis* individuals co-infected by both mites and the fungus died earlier than those infected by the fungus only (Riddick, 2010).

Hesperomyces virescens has also been used to co-infect *H. axyridis* individuals in a laboratory experiment together with *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycota: Hypocreales, Cordycipitaceae) and *Metarhizium brunneum* Petch (Sordariomycetes: Hypocreales, Calvicipitaceae) (Berry, 2017; Haelewaters et al., 2020). *Beauveria bassiana* is among the best studied of the coccinellid pathogenic fungi. Germinating spores penetrate the host's integument, which is followed by mycelial proliferation inside the host's body. The fungus feeds on the host while it is alive. Infection is usually fatal for the host; after its death *B. bassiana* becomes saprophytic and produces conidia for further transmission (Ceryngier et al., 2012). While *B. bassiana* is considered a major mortality factor for many coccinellids, *H. axyridis* has been shown to lose fecundity under laboratory conditions, but mortality was not increased (Roy et al., 2008). Experiments co-infecting *H. axyridis* with *H. virescens* together with either *B. bassiana* or *M. brunneum* have no increased mortality in comparison to infection with only *H. virescens*. In contrast, the North American-native ladybird *Olla v-nigrum* (Mulsant) (Arthropoda: Coleoptera, Coccinellidae) was more susceptible to *B. bassiana* after infection by *H. virescens* (Haelewaters et al., 2020). This differential susceptibility between the native and invasive species is in line with the enemy release hypothesis, which, as detailed above, predicts that invasive species are so successful in part because they are less susceptible to the local natural enemies compared to native species.

DISCUSSION AND FUTURE DIRECTIONS

The above inventory of various pathogens and parasites co-infecting *H. axyridis* illustrate the fundamental gaps in our understanding of how these species interact on an ecological level, and that little research examines multiple groups of pathogens at once. Despite *H. axyridis* being a widely studied model organism for invasion biology (Roy and Wajnberg, 2008), little is known about its natural enemies. Additionally, the relatively minor effects on fitness by its natural enemies, even in simultaneous infections, have underlined the resilience of this invasive alien species. Still, that does not mean looking at co-infections is a fruitless endeavor, with some simultaneous infection combinations proving mortal in laboratory experiments. The surface of this area of research has barely been scratched, and further studies are warranted.

In nature, individual hosts are frequently infected by multiple pathogens, parasites, or parasitoids. However, the effects on survival rate and usability of a combination of natural enemies as biocontrol often remains unclear. One way to improve biological control of invasive and pest species is to exploit the potentially additive function of co-infections. Therefore, we need to understand (i) what biotic and abiotic factors determine the likelihood of a co-infection on a host, (ii) what the exact biological mechanisms are that cause double infections to have an additive negative effect on host survival or fecundity, and (iii) the ways in which these interactions affect the local ecology and non-target species.

This requires an approach that is neither a specifically pair-wise interaction between two species, nor a much larger-scale population-level analysis. This includes network approaches seeking to illuminate intra- and interspecific interactions between hosts and their multiple enemies (Roy and Lawson Handley, 2012). These interactions will likely also vary under different conditions and on different spatial and temporal scales. For example, fungal ectoparasites of ladybirds have been shown to be more prevalent in urban environments (urban heat island effect; Welch et al., 2001), and simultaneous infection can be extremely localized (Raak-van den Berg et al., 2014). To not just take a shot in the dark, it is therefore useful to identify which combinations of co-infecting species already exist in nature, and draw surveys and experiments from there. Molecular data should prove enlightening for unraveling the interactions between hosts and their various natural enemies—even more so for host–parasite interactions, as parasites are often cryptic, hidden, or inside the host, and therefore difficult to track by traditional surveying methods (Hesketh et al., 2010). Another potentially valuable avenue for collecting data on novel host–parasite interactions is found in citizen science; monitoring websites such as iNaturalist can provide reports of new occurrences of interactions between a host and its parasites, and in new localities (Haelewaters et al., 2019). Citizen science projects monitoring *H. axyridis* such as the Lost Ladybug Project in the United States and the United Kingdom Ladybird Survey, among others, also generate image collections that can be screened for parasites (Fothergill et al., 2010; Brown et al., 2018; Werenkraut et al., 2020). Similarly, digitized museum collections can be screened for ectoparasites using a stereoscope relatively easily (Báthori et al., 2017; Haelewaters et al., 2017).

Harmonia axyridis makes for a powerful model organism, both as an invasive alien species and for the study of simultaneous infections by natural enemies. While spreading around the world, to every continent except Australia and Antarctica, *H. axyridis* may have brought its natural enemies with it and it has interacted with a wide range of locally native organisms. As a common factor among all those locations, this globetrotter ladybird makes for a great study subject on global ecological interactions.

AUTHOR CONTRIBUTIONS

DH: conceptualization. MG: writing—original draft and visualization. MG and DH: writing—review and editing. Both authors have read and agreed to the published version of the manuscript.

FUNDING

DH is supported by the Research Foundation–Flanders (Junior Postdoctoral Fellowship 1206620N) and the Czech Science Foundation (grant 21-06446S).

ACKNOWLEDGMENTS

The first author would like to thank the continually inspiring Menno Schilthuizen (Naturalis Biodiversity Center and Taxon Expeditions, Leiden, the Netherlands), whose suggestion of the second author as a supervisor has resulted in an extraordinarily interesting pandemic year. The second author wants to take the opportunity to thank Ted E. Cottrell (Agricultural Research Service, Southeastern Fruit and Tree Nut Research Laboratory, U.S. Department of Agriculture, Byron, Georgia) for his enduring generosity and mentorship since the spring of 2014.

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