



Infection patterns of *Harmonia axyridis* (Coleoptera: Coccinellidae) by ectoparasitic microfungi and endosymbiotic bacteria

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ABSTRACT

The invasive alien ladybird *Harmonia axyridis* (Coleoptera: Coccinellidae) hosts a wide range of natural enemies. Many observations have been done in nature but experimental studies of interactions of multiple enemies on *Ha. axyridis* are rare. In light of this knowledge gap, we tested whether the host phenotype and presence of bacterial endosymbionts *Spiroplasma* and *Wolbachia* affected parasitism of *Ha. axyridis* by the ectoparasitic fungus *Hesperomyces harmoniae* (Ascomycota: Laboulbeniales). We collected 379 *Ha. axyridis* in the Czech Republic, processed specimens, including screening for *He. harmoniae* and a molecular assessment for bacteria, and calculated fecundity and hatchability of females. We found that high hatchability rate (71 %) was conditioned by high fecundity (20 eggs daily or more). The average parasite prevalence of *He. harmoniae* was 53 %, while the infection rate of *Spiroplasma* was 73 % in ladybirds that survived in winter conditions. *Wolbachia* was only present in 2 % of the analyzed ladybirds. Infection by either *He. harmoniae* or *Spiroplasma* did not differ among host color morphs. In the *novemdecimsignata* morph, younger individuals (with orange elytra) were more heavily parasitized compared to old ones (with red elytra). Fecundity and hatchability rate of females were unaffected by infection with either *He. harmoniae* or *Spiroplasma*. However, female ladybirds co-infected with *He. harmoniae* and *Spiroplasma* had a significantly lower fecundity and hatchability compared to females with only one or no symbiont.

1. Introduction

Predaceous ladybirds are important as natural enemies in biological control of agricultural pests, particularly aphids, coccids, aleyrodids, and psyllids (Hemiptera: Sternorrhyncha) (Hodek and Honěk, 2009; Hodek et al., 2012). Ladybirds themselves are impacted by several natural enemies, including parasites, parasitoids, pathogens, and predators (Ceryngier et al., 2012, 2018; Haelewaters et al., 2017b, 2020). Several genera of intracellular bacteria cause various negative effects in ladybirds, such as killing of males (Majerus and Hurst, 1997). On the other hand, some bacteria living in insect cells have been shown to help protect against natural enemies (Hrček et al., 2018).

Laboulbeniales microfungi (Ascomycota: Laboulbeniomycetes) include both ectobionts and obligate ectoparasites that are associated with many different arthropods, mainly beetles (Coleoptera) (Haelewaters et al., 2021, 2022b; Weir and Hammond, 1997). Ladybirds (family Coccinellidae) are infected by several species of the genus

Hesperomyces and a single species of *Laboulbenia*, *L. coccinellidicola* (Haelewaters and De Kesel, 2017; Haelewaters et al., 2017a). The most commonly reported species of Laboulbeniales on ladybirds, *Hesperomyces virescens*, is among the species in the order that produce a haustorium, which penetrates the host integument to make contact with the haemocoel for nutrition (Weir and Beakes, 1996). *Hesperomyces virescens* is a species complex (SC), and includes multiple species segregated by host and geography (Haelewaters et al., 2018a; Van Caenegem et al., 2023). Thus far, in addition to *He. virescens* sensu stricto that is strictly associated with *Chilocorus stigma* (Thaxter, 1891), three species have been formally described within the complex. These are *He. halyziae* on *Halyzia sedecimguttata* (Haelewaters and De Kesel, 2020), *He. harmoniae* on *Harmonia axyridis* (Haelewaters et al., 2022c), and *He. perexochomi* on *Parexochomus nigripennis* and *Parex. quadriplagiatus* (Crous et al., 2021). Henceforth, we refer to *He. virescens* in its broad sense as *He. virescens* SC. Negative fitness effects of *He. virescens* SC on their ladybird hosts have been documented including reduced survival

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under winter conditions (Riddick, 2010) and significantly increased mortality after 14 days following infection compared to uninfected ladybirds (Haelewaters et al., 2020). Heavy infections can supposedly impede flight, mating, foraging, and feeding (Nalepa and Weir, 2007).

Most insects are associated with intracellular endosymbiotic bacteria; many of them help the insect immune system (Eleftherianos et al., 2013). Primary endosymbionts have an obligatory relationship with the insect host (the host cannot survive without them), providing essential amino acids. Often these endosymbionts show phylogenetic congruence with their hosts. Secondary endosymbionts are facultative, meaning that they are not required for successful growth or reproduction, and they are both vertically and horizontally transmitted (Chiel et al., 2009).

Secondary endosymbionts, such as *Rickettsia*, *Hamiltonella*, *Regiella*, *Serratia*, and *Wolbachia* can provide nutrients to insect hosts (Brownlie et al., 2009; Koga et al., 2003), confer resistance to parasitic wasps and fungal or viral pathogens (Oliver et al., 2003; Scarborough et al., 2005; Teixeira et al., 2008; Wong et al., 2011), and improve tolerance to heat stress (Montllor et al., 2002). At the same time, secondary endosymbionts, including *Rickettsia*, *Spiroplasma*, and *Wolbachia*, are likely to be parasitic rather than beneficial to their insect hosts. They manipulate insect reproduction by forcing asexuality, killing males, feminizing genetic males, and inducing cytoplasmic incompatibility together with parthenogenesis, with apparent selfish effect of assisting the spread of their infections into host populations (Engelstädter and Hurst, 2009; Werren and Windsor, 2000; Werren et al., 2008).

Research on endosymbiotic bacteria is hampered by the difficulty of culturing them outside of their original host. The proportion of infection in host populations varies; incidence among species in species-rich taxa tend to be higher compared to species-poor groups: e.g., 52 % [range: 48–57 %] of arthropod species are infected with *Wolbachia*, 24 % [20–42 %] with *Rickettsia*, and 13 % [13–55] with *Cardinium* (Weinert et al., 2015). Duron et al. (2008) reported heritable bacterial endosymbionts in 14 ladybird species. Among these, five species were infected by two bacterial species, whereas *Adalia bipunctata* was infected by three bacterial species. Currently, the male-killing effect has been shown for four genera of intracellular bacterial endosymbionts of ladybirds. *Rickettsia* sp. induces male-killing effects in *A. bipunctata* and *A. decempunctata* (von der Schulenburg et al., 2001; Werren et al., 1994). *Wolbachia* sp. induces male-killing in *A. bipunctata* (Hurst et al., 1999a). *Spiroplasma* sp. induces it in *A. bipunctata*, *Anisosticta novemdecimpunctata*, and *Harmonia axyridis* (Hurst et al., 1999b; Tinsley and Majerus, 2006; Zakharov et al., 1999). And, finally, *Flavobacterium* sp. induces male-killing in *Coleomegilla maculata* and *Hippodamia variegata* (Hurst et al., 1999c).

The insect immune system consists of a wide variety of defence mechanisms that act individually or in combination to prevent foreign organisms from entering the insect body or to suppress the growth and replication of pathogens once they gain access to host tissues. The first line of defence is represented by the insect epithelia, which serve as a barrier against biotic and abiotic factors, and produce local antimicrobial peptides upon infection or wounding (Davis and Engström, 2012). The second line of defence in insects is represented by the innate immune system that responds through a series of mechanisms, including alkaloids (Röhrich et al., 2012) and anti-oxidative enzymes (Awad et al., 2021).

Harmonia axyridis (Coleoptera, Coccinellidae) is a ladybird that is native to Asia, and now present as an invasive alien species on all continents except mainland Australia and Antarctica (Hiller and Haelewaters, 2019; Roy et al., 2016). Originally a beneficial biological control agent, it is now a model organism for studying invasive alien species (Roy and Wajnberg, 2008). While it possesses potent immunity mechanisms (Schmidtberg et al., 2013), *Ha. axyridis* has been reported as a host to various natural enemies: pathogens (bacteria, endobiotic fungi, protists), parasites (*Hesperomyces harmoniae* ectoparasitic fungi, *Coccipolipus hippodamiae* mites, *Parasitylenchus bifurcatus* nematodes), parasitoids (phorid and tachinid flies, *Dinocampus coccinellae*, *Homalotylus*,

and Tetrastichinae wasps), and predators (bugs, lacewings, ladybirds, spiders, and birds) (Ceryngier et al., 2018; Haelewaters et al., 2017b; Harding et al., 2011; Raak-van den Berg et al., 2014; Riddick et al., 2009).

Natural enemies of ladybirds have recently, after several years of delay, started using invasive populations of *Ha. axyridis* as a new host in various geographic areas (Ceryngier et al., 2018; Garcés and Williams, 2004; Haelewaters et al., 2017b; Knapp et al., 2019; Raak-Van den Berg et al., 2014). When acting alone, they have only a limited potential for controlling ladybird populations (Ceryngier et al., 2018; Haelewaters et al., 2017b; Riddick, 2010; Roy et al., 2008). And while double infections of *Olla v-nigrum* with *He. virescens* SC and either *Beauveria bassiana* or *Metarhizium brunneum* significantly increase mortality of ladybirds compared to infection by only *He. virescens* SC, mortality is not affected by such double infections in *Ha. axyridis* (Haelewaters et al., 2020). In North America, the density of parasitic mites is higher on fungus-infected beetles than on fungus-free beetles, and vice versa (Riddick, 2010). The abundance of *Spiroplasma* in samples of *Ha. axyridis* with a single strain of this bacterium is 10× lower than in samples with multiple infections (Goryacheva et al., 2018). All known double infections of *Ha. axyridis* were recently reviewed by de Groot and Haelewaters (2022).

The aim of this study was to test whether the host phenotype and presence of bacterial endosymbionts, suspected as male killers, affect (i) parasitism of invasive ladybirds by *He. harmoniae* and (ii) host fitness.

2. Materials and methods

2.1. Sampling

We collected six samples of *Ha. axyridis* ladybirds in four localities in the Czech Republic: South Bohemian Region, České Budějovice, 48°59'N 14°27'E, on building walls, 28 September 2017, during migration to overwintering sites, 90 specimens (sample CBA); *ibid.*, 29 September 2017, 51 specimens (sample CBB); South Bohemian Region, near České Budějovice, 48°59'N 14°27'E, on ornamental shrubs, 16 June 2018, during the breeding season, 126 specimens (sample CBS); South Bohemian Region, Horní Dvoriště, 48°36'N 14°22'E, on teepee, 30 September 2017, during migration, 24 specimens (sample HD); South Bohemian Region, Hvozdná, 49°09'N 14°33'E, inside a house, 6 November 2017, after migration/during overwintering, 34 specimens (sample HV); Central Bohemian Region, Srbsko, 49°57'N 14°07'E, on limestone rock walls, 29 September 2017, during migration, 54 specimens (sample SR). Ladybirds from all samples except CBS were placed in 1-L glass jars and provided with moist cotton and corrugated dry filter paper. The jars were ventilated through nylon mesh. All jars with autumn samples were placed for overwintering in an air-conditioned room at 5 °C and photoperiod of 12:12 (L:D). Every month, jars were taken to the laboratory at room temperature, water was replenished, and ladybirds were allowed to warm up and provided water (Awad et al., 2013). Ladybirds from sample CBS were processed immediately, without prolonged cold storage.

2.2. Processing of specimens

In June 2018, overwintering samples were relocated to the laboratory. After 24 h, living and dead individuals were sorted out, and every specimen received a unique code. Living and dead specimens were sexed and assigned a color morph (*novemdecimsignata* = *succinea*, *spectabilis*, *conspicua*; sensu Roy et al., 2016) using a stereomicroscope at 10× magnification. For *novemdecimsignata* specimens (except in CBA and HV samples), we categorized carotenoid coloration [orange (O), red (R)] and described whether spots were well-circumscribed, separate, and 19 in number (0); missing or fewer in number than 19 (–), or large and touching each other (+) (Fiedler and Nedvěd, 2019; Haelewaters et al., 2022a). All ladybird specimens were screened for parasitism with *He.*

harmoniae by looking for three-dimensional thalli (Blackwell et al., 2020; Fig. 1) in six body zones (sensu Fiedler and Nedvĕd, 2019): head (1), pronotum (2), elytra (3), thoracic sterna (4), abdominal sterna (5), legs (6). We use the term “parasite prevalence” for the proportion of host specimens parasitized by *He. harmoniae*, and “infection rate” for the bacteria.

2.3. Fecundity and fertility assessment

Living female ladybirds were placed individually in Petri plates and reared on *Acyrtosiphon pisum* (pea aphids) raised on faba bean seedlings in a Sanyo MIR 153 incubator at different temperatures for 6-hour intervals, i.e., 20 °C, 25 °C, 20 °C, 15 °C, and photoperiod of 18:6 (L:D). During the following two weeks, the first five egg clusters were separated from the Petri plates in which the ladybirds were reared. The number of eggs and hatched larvae were counted, the average number of eggs per cluster and the hatchability rate were calculated. During this breeding period, all specimens were observed for the possible appearance of insect parasitoids such as *D. coccinellae*.

2.4. Endosymbiont assessment

DNA was extracted from females from all autumn samples (CBA, CBB, HD, and SR) using QIAmp DNA Mini Kit (QIAGEN, Hilden, Germany). For *Rickettsia*, we amplified the *gltA* gene using primers RicF141 (5′-TCGGTTCCTTTTCGGCATTTTA-3′) and RicR548 (5′-GCATATTTATCACCCTTCATT-3′) (Goryacheva et al., 2017). For *Spiroplasma*, we amplified the 23S subunit of the ribosomal RNA gene using primers SP-ITS-JO4 (5′-GCCAGAAGTCAGTGTCTAACC-3′) and SP-ITS-N55 (5′-ATTCCAAGCCATCCACCATACG-3′) (Goryacheva et al., 2017). For *Wolbachia*, we amplified the we used surface protein gene *wsp* using primers Wsp_F1 (5′-GTCCAATARSTGATGARGAAAC-3′) and Wsp_R1 (5′-CYGCACCAAYAGYRCTRTRAAA-3′) (Baldo et al., 2006). PCRs were run in 10- μ l reactions containing 5 μ l of Combi PPP Master Mix (Top-Bio, Vestec, Czech Republic), 5 pmol of each primer, and ~ 50 ng of DNA extract. Cycling conditions were as follows: For *Rickettsia*: initial denaturation at 95 °C for 3 min; followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 45 s, and extension at 72 °C for 60 s; and final extension at 72 °C for 10 min. For *Spiroplasma*: initial denaturation at 95 °C for 4 min; followed by 38 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 40 s, and extension at 72 °C for 40 s; and final extension at 72 °C for 5 min. For *Wolbachia*: initial



Fig. 1. Thalli of *Hesperomyces harmoniae* at the right elytral tip of *Harmonia axyridis*. Scanning electron micrograph by Samane Sakaki (University of South Bohemia).

denaturation at 95 °C for 3 min; followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min; and final extension at 72 °C for 10 min.

PCR products were loaded onto TAE 1.5 % agarose gels for electrophoresis at 100 V. The presence of a single band on a gel was seen as a confirmation of the presence of the corresponding bacterium in the ladybird (Fig. 2).

2.5. Statistical analysis

The Mann-Whitney *U* test was used to compare the number of parasitized body zones between two groups of data whereas the Kruskal–Wallis test was used for comparison among three groups. A Yates-corrected Pearson’s chi-square test ($df = 1$) was used for 2 \times 2 frequency tables. A classic Pearson’s chi-square test was needed for the analysis of parasite prevalence of individual body zones. A one-way analysis of variance (ANOVA) was used to show effects of diverse factors on fecundity and fertility. Parameters of logistic regression of the increase of hatching rate with fecundity were estimated using the nonlinear estimation Levenberg–Marquardt method. All statistical analyses were performed in STATISTICA 13 (TIBCO Software, Palo Alto, California, USA).

3. Results

3.1. Survival rate, sex ratio, and color morphs

The survival rates from October 2017 to June 2018 were low for both samples CBA and HV and the surviving ladybird specimens were in bad condition (i.e., slow movements, no attempts to try at room temperature); these specimens were not further analyzed. All individuals of the CBS sample, collected in June 2018, were alive. Samples CBB, HD, and SR had survival rates between 33 % and 45 % (Table 1), with the survival rate of females being significantly higher than of males (Yates-corrected $\chi^2 = 7.55$, $p = 0.006$).

The sex ratio (male percentage) of the samples varied from 37 % to 61 % (Table 1) but, in general, did not deviate from the expected 50:50 ratio (unweighted average of sample sex ratios = 47 %, total sex ratio = 50 %). However, there was a significant difference between the sex ratio in the spring sample (CBS: 61 %) and the joint autumn samples (CBA, CBB, HD, HV, and SR: 45 %; Yates-corrected $\chi^2 = 8.23$, $p = 0.004$). The proportion of melanic morphs (*spectabilis* + *conspicua*) was 7 %, with no seasonal change; only males were found of the *conspicua* morph ($n = 4$, 1 % of total specimens).

3.2. Parasitism by *Hesperomyces harmoniae* and *Dinocampus coccinellae*

A total of 170 ladybirds were parasitized by *He. harmoniae*, resulting in an average parasite prevalence of 53 % for the six samples. Parasite prevalence ranged among samples from 27 % to 91 % (Table 2). The spring sample (CBS: 27 %) had a significantly lower parasite prevalence compared to the average prevalence of the joint autumn samples (CBA, CBB, HD, HV, and SR: 54 %; Yates-corrected $\chi^2 = 23.3$, $p < 0.0001$). Of the three autumn samples that had viable survivors by June 2018 (CBB, HD, SR), dead ladybirds were significantly more parasitized by *He. harmoniae* (75 %) than living ones (28 %; Yates-corrected $\chi^2 = 44.4$, $p < 0.0001$). Parasite prevalence of females (44 %) was similar to that of males (46 %; Yates-corrected $\chi^2 = 0.04$, $p = 0.84$).

The average number of parasitized body zones, i.e., body zones on which thalli of *He. harmoniae* were observed, was in general 0.79 per ladybird (Table 2). This number was significantly lower in the spring sample (0.40) than in the joint autumn samples (0.98; Mann–Whitney $Z = 4.76$, $p = 2 \cdot 10^{-6}$). Even when only analyzing parasitized individuals, more zones were parasitized on dead individuals (1.87) than on living ones (1.25; Mann–Whitney $Z = 2.95$, $p = 3 \cdot 10^{-3}$). The number of parasitized zones per individual ladybird did not significantly differ between

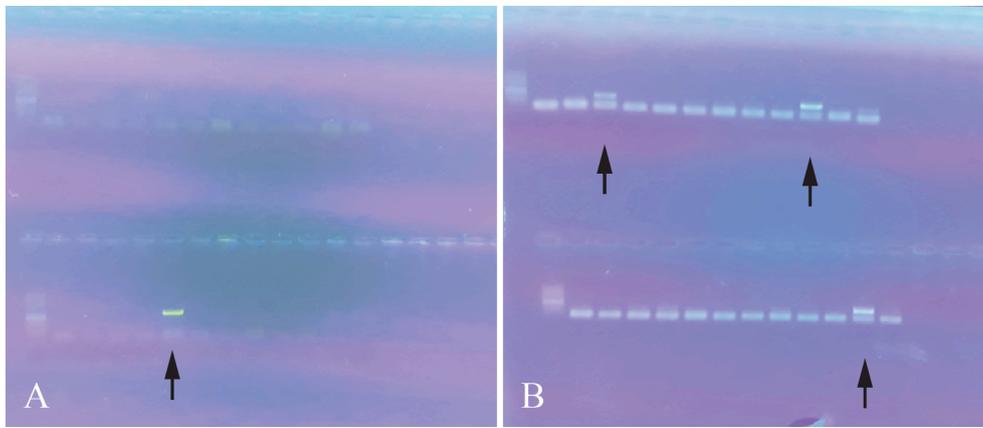


Fig. 2. Agarose gels showing specific bands (arrows) confirming the presence of two bacteria in *Harmonia axyridis* (sample CBA). **A.** *Wolbachia*, one individual positive. **B.** *Spiroplasma*, three individuals positive.

Table 1

Parameters of the analyzed samples of *Harmonia axyridis*. Abbreviations (units): SuR, survival rate (%); SeR, sex ratio (% of males); F, females; M, males; Nov: morph *novemdecimsignata* (number of females/males); Spe, morph *spectabilis* (number of females/males); Con, morph *conspicua* (number of females/males); Fec ± stdev, fecundity (average daily number of eggs laid by a female) ± standard deviation; Hatch ± stdev, hatchability rate of the eggs (%) ± standard deviation.

Sample	SuR	SuR F	SuR M	SeR	Nov	Spe	Con	Fec ± stdev	Hatch ± stdev
CBA	–	–	–	37	50/30	7/2	0/1	–	–
CBB	45	65	29	55	21/25	2/3	0/0	32 ± 11	74 ± 7
CBS	–	–	–	61	47/71	2/3	0/3	24 ± 14	51 ± 33
HD	33	27	38	54	11/13	0/0	0/0	23 ± 24	25 ± 28
HV	–	–	–	38	19/13	2/0	0/0	–	–
SR	37	50	15	37	32/19	2/1	0/0	13 ± 11	10 ± 26
Total	–	47 ^a	27 ^a	47 ^a	180/171	15/9	0/4	23 ± 15 ^b	44 ± 35 ^b

^a Unweighted average of the meaningful sample values.

^b Average ± standard deviation of individual values.

Table 2

Parasitism of *Harmonia axyridis* by *Hesperomyces harmoniae*. Abbreviations (units): HespP, parasite prevalence = proportion of host specimens parasitized by *He. harmoniae* (%); Zones (average number of body zones with thalli per ladybird); HespPD, parasite prevalence of dead individuals (%); HespPL, parasite prevalence of living individuals (%). Infection of *Ha. axyridis* by bacteria. Abbreviations (units): SpirD, infection rate by *Spiroplasma* in dead individuals (%); SpirL, infection rate by *Spiroplasma* in living individuals (%); Wolb, infection rate by *Wolbachia* (%).

Sample	HespP	Zones	HespPD	HespPL	SpirD	SpirL	Wolb
CBA	29	0.36	(29)	–	19	–	2
CBB	55	0.96	79	26	–	77	0
CBS	27	0.40	–	(27)	–	–	–
HD	38	0.42	50	13	–	67	0
HV	91	2.29	(91)	–	13	–	0
SR	78	1.46	97	45	–	80	7
Total	53 ^a	0.79	75 ^a	28 ^a	16 ^a	75 ^a	2 ^a

^a Unweighted average of the comparable values, without the odd values in parentheses.

females (0.73) and males (0.85; Mann–Whitney $Z = 0.67$, $p = 0.51$). There was, however, a significant difference between females and males in the infection of individual body zones ($\chi^2(5) = 23.6$, $p = 0.00026$): head and elytra were more parasitized in females, whereas the other body zones were more parasitized in males (Table 3).

There was no significant difference among color morphs in their parasite prevalence (Yates-corrected $\chi^2(2) = 0.32$, $p = 0.56$) nor in the number of body zones with thalli (Kruskall–Wallis $\chi^2(2) = 1.94$, $p = 0.38$). In the *novemdecimsignata* morph, orange individuals had a higher probability of infection (Yates-corrected $\chi^2(1) = 2.07$, $p = 0.038$) and number of body zones with thalli (0.89) compared to red individuals

Table 3

Parasite prevalence (%) of individual body zones over all samples of *Harmonia axyridis*. Body zones: 1, head; 2, pronotum; 3, elytra; 4, thoracic sterna; 5, abdominal sterna; 6, legs. F, females; M, males.

Sex	1	2	3	4	5	6
F	11	13	37	6	1	5
M	8	18	32	12	5	11

(0.57; Mann–Whitney $Z = 5.85$, $p = 0.016$). Individuals with different size of spots did not differ in the number of body zones with thalli observed (Kruskall–Wallis $\chi^2(2) = 1.46$, $p = 0.48$).

Dinocampus coccinellae emerged from two females and three males of the CBS sample (all *novemdecimsignata* morph, none with *He. harmoniae*). No emergence was observed from ladybirds of the autumn samples.

3.3. Infection rate

For *Spiroplasma*, the infection rate of analyzed females was on average 16 % in the two samples with dead individuals (CBA, HV) and 75 % in the three autumn samples with viable surviving individuals (CBB, HD, SR) (Table 2). There were on average 38 % *Spiroplasma*-infected *novemdecimsignata* females and 50 % *Spiroplasma*-infected *spectabilis* females. These infection rates among color morphs were not significantly different (Yates-corrected $\chi^2 = 0.02$, $p = 0.89$). Among the living *novemdecimsignata* females, the infection rate of orange and red individuals with *Spiroplasma* was not significantly different (75 % versus 80 %; Yates-corrected $\chi^2 = 0.11$, $p = 0.74$). We did not detect *Rickettsia* in any of the analyzed females. Finally, *Wolbachia* was detected only in

two individuals (infection rate of 2 %), one in a dead female without *Spiroplasma*, one in a living female with *Spiroplasma*.

Cross-tabulation of analyzed females with or without *He. harmoniae* and with or without *Spiroplasma* (Hesp0Spir0: 34, Hesp1Spir0: 19, Hesp0Spir1: 22, Hesp1Spir1: 12) showed that the two infections were independent of each other (Yates-corrected $\chi^2 = 0.03$, $p = 0.86$). The same occurred for the number of body zones with *He. harmoniae* thalli on females with or without *Spiroplasma* (Mann–Whitney $Z = 0.17$, $p = 0.87$).

3.4. Fecundity and hatchability

The average daily number of eggs laid by a female (fecundity) regardless of sample was 22. The proportion of eggs that hatched to a larva (fertility rate, hatchability) was 43 %. Both these parameters were higher for samples CBB (autumn) and CBS (spring) than for autumn samples HD and SR (Table 1). There was a strong correlation between individual fecundity and hatching rate, although not linear. Hatching rate increased dramatically from almost 0 % to 71 % at a fecundity of 20 (Fig. 3); when females laid <20 eggs, the majority of those eggs did not hatch.

The fecundity of females infected by *Spiroplasma* was lower (18 eggs) than that of uninfected females (30 eggs) but the difference was not significant (ANOVA $F_{(1,28)} = 3.77$, $p = 0.06$). The hatchability rate of females infected by *Spiroplasma* was lower (0.33) than that of uninfected females (0.47) but, again, the difference was not significant ($F_{(1,28)} = 0.75$, $p = 0.39$). Likewise, the fecundity of females parasitized by *He. harmoniae* was lower (19 eggs) than that of females without *He. harmoniae* (23 eggs), but the difference was not significant ($F_{(1,55)} = 1.01$, $p = 0.32$). Finally, the hatchability rate of females with and without *He. harmoniae* was equal (0.43; $F_{(1,55)} = 0.003$, $p = 0.96$). A synergistic effect of fungal parasitism and bacterial infection in the same female resulted in a significantly lower fecundity (12 eggs) compared to females with no or just one of the symbionts (32 eggs; $F_{(1,28)} = 22.04$, $p = 6 \cdot 10^{-5}$). Also the hatching rate was lower in the double-infected females (0.19) than in the females with no or only one symbiont (0.59; $F_{(1,28)} = 11.68$, $p = 0.002$).

There was lower fecundity of females of the *novemdecimsignata* morph with orange elytra (18 eggs) compared to those with red elytra

(27 eggs; $F_{(1,53)} = 5.56$, $p = 0.02$). There was also a lower hatchability of eggs laid by females of the *novemdecimsignata* morph with orange elytra (0.32) compared to those with red elytra (0.56; $F_{(1,53)} = 6.72$, $p = 0.01$). This was caused by the spring sample CBS, of which all five orange females had very low fecundity (1–12 eggs) and hatchability rate (0). There was a trend for the females of the *novemdecimsignata* morph with larger spots to have higher fecundity (17 eggs for ladybirds categorized as “–”, 22 for “0”, 30 for “+”), but the difference was not significant ($F_{(2,52)} = 0.52$, $p = 0.60$) due to high variability. Hatchability did not depend on degree of melanization ($F_{(2,52)} = 0.76$, $p = 0.48$).

4. Discussion

4.1. Parasite prevalence

The average parasite prevalence by *He. harmoniae* of all our samples of *Ha. axyridis* was 53 %. For the České Budějovice samples, the overall prevalence was 28 % ($n = 267$; samples CBA, CBB, and CBS). Excluding the spring sample (CBS), the overall prevalence on ladybirds collected in České Budějovice was 38 % ($n = 141$). In earlier studies during which *Ha. axyridis* specimens were collected during autumn flight in České Budějovice, the parasite prevalence ranged from 19 % ($n = 486$, collected in 2018; Haelewaters et al., 2022a) to 26 % ($n = 1102$, collected in 2014; Fiedler and Nedvěd, 2019). These numbers confirm previous observations that parasitism of *Ha. axyridis* by *He. harmoniae* varies significantly over time, which may be the result of a combination of biotic and abiotic variables (Haelewaters et al., 2017b, 2022a; Raak-van den Berg et al., 2014).

Dead individuals from the three autumn samples that had viable survivors (CBB, HD, SR) were more parasitized (prevalence = 75 %) compared to living individuals from these samples (28 %). In addition, more body zones with thalli of *He. harmoniae* were found on dead individuals (1.87) than on living ones (1.25). In North American laboratory assays, the mortality of *He. harmoniae*-parasitized *Ha. axyridis* was significantly higher than that of specimens without *He. harmoniae*; >50 % of parasitized ladybirds had died after 14 days (Haelewaters et al., 2020). A recent study found that winter survival of *Ha. axyridis* parasitized by *He. harmoniae* was significantly lower compared to

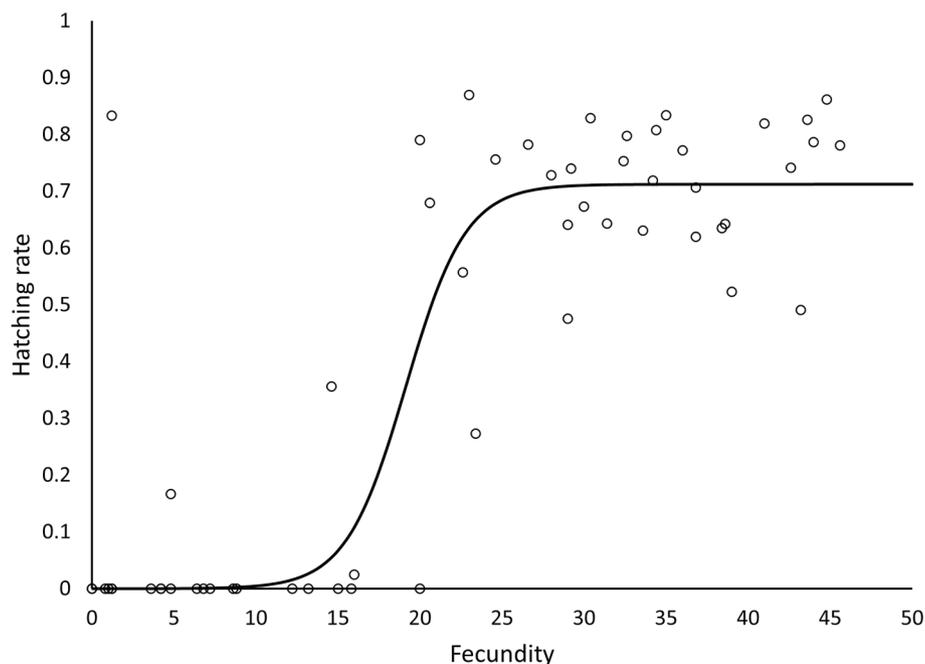


Fig. 3. Logistic regression of the increase of hatchability rate with fecundity: $y = f \cdot e^{a+b \cdot x} / (1 + e^{a+b \cdot x})$, with $f = 0.71 \pm 0.03$, $a = -10 \pm 5$, $b = 0.55 \pm 0.23$, $R^2 = 0.76$, $F = 171$, and $p < 10^{-6}$.

unparasitized ladybirds and that body mass loss during overwintering was higher for parasitized individuals compared to unparasitized ones (Knapp et al., 2022). There is a real mortality cost for *Ha. axyridis* when parasitized by *He. harmoniae*.

Parasitism of male and female ladybirds by *He. harmoniae* was similar, but females were more infected on their head and elytra whereas males bore more thalli on the other body zones (pronotum, legs, sterna). During mating, males make vibration movements while having their ventral side on top of the female elytra. This is in line with previous findings that *He. virescens* SC can be considered a sexually transmitted disease (Welch et al., 2001). Note that during overwintering, parasite prevalence of *He. harmoniae* on *Ha. axyridis* typically increases in non-sex-related patterns suggesting social transmissions through physical contact (Nalepa and Weir, 2007; Riddick, 2006; Riddick and Schaefer, 2005).

We hypothesized that older ladybirds would have a higher probability to be parasitized by *He. harmoniae*, due to more opportunities for spore transmission. We know that carotenoid accumulation (red color intensity) is a function of ladybird age (Nedved et al., 2019), and Haelewaters et al. (2022a) previously reported that *Ha. axyridis* individuals with red elytra were more likely and individuals with yellow elytra less likely to be parasitized by *He. harmoniae* compared to individuals with orange elytra. This was explained by physical contact (during mating and overwintering) as well as grooming, both of which make that parasite prevalence and thallus density are positively correlated with age (~ elytral color). However, in our study, orange (younger) individuals had a higher number of body zones with thalli (0.89) compared to red (older) individuals (0.57). A previous study found that parasitized *Ha. axyridis* ladybirds had increased elytral carotenoid contents (~ age) and that this effect was more pronounced with increasing mass (Fiedler and Nedved, 2019). We think that among the old ladybirds, many parasitized individuals already died due to parasitism with *He. harmoniae* (but large individuals survived even with the parasite), whereas the younger parasitized individuals were more likely to be still alive, because of the mild and delayed effect of the parasite.

Dinocampus coccinellae wasps emerged from five ladybirds of the spring sample (CBS), which accounts for a prevalence of 5%. No wasps emerged from ladybirds of any of the autumn samples. In comparison, in 2015–2018, in several sites in Czech Republic, the average parasite prevalence of *D. coccinellae* on *Ha. axyridis* was 12.6%, similar to the native *Coccinella septempunctata* (12.2%; Knapp et al., 2019).

4.2. Infection rate

The infection rate by *Spiroplasma* was 75% in the surviving females of the autumn samples. Li et al. (2021) recorded *Spiroplasma* in *Ha. axyridis* in 15 of 30 localities within the native range (China, Japan, and Siberia) on average in 10% of individuals, but not in any of eight invasive North American populations. The infection rate for *Spiroplasma* was much lower in dead individuals compared to living ones. This might be explained by bad preservation, and thus detection possibility, of bacteria in old dry insects.

We found *Wolbachia* in only two females. We consider these “accidental” infections. In China, *Wolbachia* was found only in one of 30 examined localities, whereas in North America it was recorded in three of eight localities and in high percentage (Li et al., 2021). Although present in other ladybird species, *Rickettsia* was not found in our study. It was also absent from native and invasive populations in the study by Li et al. (2021).

The two infections of ladybirds (*He. harmoniae* and *Spiroplasma*) were independent of each other. Thus, the endosymbiotic bacterium neither weakened its host to nor protected it from parasitism by *He. harmoniae*, and vice versa. None of the ladybirds parasitized by *D. coccinellae* wasps were simultaneously parasitized by *He. harmoniae*, but this exclusion may have appeared by chance because of the low number of ladybirds with *D. coccinellae*. Similarly, Orlova-Bienkowskaja et al. (2018) did not

find a correlation between parasitism of *Ha. axyridis* by *He. harmoniae* and infection by the parasitic nematode *Parasitylenchus bifurcatus*. Raakvan den Berg et al. (2014) found that *Paras. bifurcatus*-infected ladybirds were significantly more likely to be parasitized by *He. harmoniae* than ladybirds without nematodes (in Tilburg). Laboratory assays in North America found no additive effect to mortality of *Ha. axyridis* ladybirds when first parasitized with *He. harmoniae* and then exposed to entomopathogenic fungi (*Beauveria bassiana* or *Metarhizium brunneum*), contrary to the North American-native ladybird *Olla v-nigrum*, which showed significantly higher mortality rates when co-infected by both *He. virescens* SC and either entomopathogenic fungus (Haelewaters et al., 2020). The full suite of interactions among *Ha. axyridis* and its natural enemies is not well understood; more large-scale bioassays as well as standardized monitoring studies are needed (sensu Haelewaters et al., 2020; Soares et al., 2022).

4.3. Ladybird survival

We saw high survival rates (33% to 45%) of autumn samples of *Ha. axyridis* after prolonged cold storage (eight months at 5 °C, with each month an interruption of several hours at laboratory temperature), comparable to our previous study (Awad et al., 2013). This is a desirable feature of biological control agents, which need to be available in large quantities at any given time (Leopold, 2007). Studies using laboratory reared ladybirds often show much reduced survival—e.g., two weeks at 4 °C to 10 °C in *A. decempunctata* (Mojib-Haghighadam et al., 2022), 45 days at 6 °C in *Coccinella undecimpunctata* (Abdel-Salam and Abdel-Baky, 2000). Alternating temperatures and regular short high-temperature interruptions during cold storage are beneficial for various stored insects (Colinet et al., 2015; Sakaki et al., 2019). Likewise, we suggest to introduce monthly high-temperature interruptions during long-term cold storage of *Ha. axyridis* for optimal ladybird survival.

4.4. Reproduction and the effect of co-infections

The average daily fecundity (22 eggs) that we observed is typical for many medium- and large-sized aphidophagous ladybirds (Awad et al., 2013; Nedved and Honek, 2012), while the average hatchability rate was 43%, which is below the usual reproductive capacity of *Ha. axyridis* (85% in Awad et al., 2013). The low hatchability rate was in fact a combination of almost no hatching in some of the females that also had low fecundity, and over 70% hatching in “healthy” females with high fecundity (>20 eggs). Hatchability rates below 60% in aphidophagous ladybirds in laboratory studies should be viewed as suspicious and factors causing non-fertilization or embryonic death should be considered.

While *He. harmoniae* and *Spiroplasma* did not significantly affect fecundity of females or hatchability of eggs, the synergistic effect of fungal parasitism and bacterial infection in the same female (type B interaction; de Groot and Haelewaters, 2022) resulted in significantly lower fecundity (12 versus 32 eggs) and hatchability (19% versus 59%) than in females with no or just one of the symbionts. Studies focusing on the influence of co-infections on the fecundity of hosts are rare, which makes it difficult to draw general patterns. The fecundity of female *Nasonia vitripennis* wasps co-infected with two strains of *Wolbachia* was significantly higher compared to that of uninfected females (Bordenstein and Werren, 2000). On leaflets of cultivated and wild-type tomato plants, *Tetranychus truncatus* spider mites that were co-infected with *Spiroplasma* and *Wolbachia* deposited a higher number of eggs compared to uninfected mites, an effect not observed in spider mites on plants suppressed in their defense response (Zhu et al., 2020). It is interesting that in these two studies, the fecundity as a result of co-infection was increased, whereas the fecundity of *Ha. axyridis* ladybirds co-infected with *He. harmoniae* and *Spiroplasma* was decreased. The difference may lie in the fact that the studies of Bordenstein and Werren (2000) and Zhu et al. (2020) focused on two endosymbionts. In our study, one of the symbionts (*He. harmoniae*) is an ectoparasite that penetrates the host

integument through a rhizoidal haustorium (Weir and Beakes, 1996). When studying co-infections, we also need to consider mechanisms of infection. In the case of *He. harmoniae*, this includes spore attachment, penetration of the integument, and development of a thallus that may be dependent on environmental factors (sensu De Kesel 1996; Péter et al., 2022).

There is hardly any evidence for the impact of double infections on population density of *Ha. axyridis*. After the occurrence of *Ha. axyridis* in the Czech Republic in 2006 (Špryňar, 2008), no fungi nor parasitoid wasps were found in the population and the species spread rapidly through the country (Nedvěd, 2014). High parasite prevalence of *Ha. axyridis* by *He. harmoniae* in the Czech Republic was first found in 2014 (Haelewaters et al., 2017b; Fiedler and Nedvěd, 2019). The first *D. coccinellae* wasps were found in 2015 and reached high parasite prevalence in studied populations in 2016 (Ceryngier et al., 2018; Knapp et al., 2019). Note that this study marks the first time that *Ha. axyridis* ladybirds are screened for the presence of bacteria in the Czech Republic. Locally, there were less ladybirds during these years with increased parasite prevalences (O. Nedvěd et al., personal observation). However, population dynamics of *Ha. axyridis* are difficult to follow because there is a great seasonal and local variation of abundance (Honěk et al., 2018). Bottom-up control of populations through the availability of prey may also play a role in the local abundance of *Ha. axyridis* (Viglášová et al., 2017).

4.5. Color morphs

The proportion of the two melanic morphs (those with black background color of elytra, *spectabilis* + *conspicua*) that we found in surviving ladybirds (7 %) is lower than what we found in České Budějovice during migration shortly after the invasion of *Ha. axyridis* in the Czech Republic (13 %; Nedvědová et al., 2013). Honěk et al. (2020) observed a similar decrease of melanics in the population of *Ha. axyridis* over many places in Europe from 2012 to 2018. A decreased percentage of melanics (6 %) was typical for the spring cohort after overwintering—compared with melanics accounting for > 10 % of total ladybirds during autumn migration.

The color morphs in our samples did not differ in the parasite prevalence and number of body zones with thalli of *He. harmoniae*. Haelewaters et al. (2022a) analyzed 3568 *Ha. axyridis* ladybirds collected throughout Central Europe and found that the two melanic morphs had a lower parasite prevalence compared to the non-melanic *novemdecimsignata* morph. And based on 763 *Ha. axyridis* ladybirds (all of them the *novemdecimsignata* morph) collected in Massachusetts, USA, Haelewaters et al. (2018b) observed a slight trend to higher intensity of parasitism, measured in number of thalli on each ladybird, in more melanic males (categorized as “+” sensu Haelewaters et al., 2022a). Replicated laboratory assays need to be performed and the expression levels of immune genes analyzed, to test the susceptibility of various color morphs of *Ha. axyridis* to *He. harmoniae*.

5. Conclusions

In six samples totaling 379 *Harmonia axyridis* ladybirds collected in the Czech Republic, we found both the ectoparasitic fungus *Hesperomyces harmoniae* and the bacterium *Spiroplasma*. *Hesperomyces harmoniae* was on average found on 53 % of all analyzed ladybirds, while *Spiroplasma* was detected in 75 % of surviving females of our autumn samples. *Wolbachia* infection was only found in two females, and *Rickettsia* infection was not detected. Infection by either *He. harmoniae* or *Spiroplasma* did not differ among the color morphs of their host. For *He. harmoniae*, we suggest bioassays to fully understand the susceptibility of color morphs of *Ha. axyridis* to parasitism by *He. harmoniae*. Female ladybirds were more parasitized on their head and elytra whereas males were more infected on the pronotum, legs, and sterna; the fungus can be considered a sexually transmitted disease (notwithstanding in

overwintering aggregations it transmits socially). *Spiroplasma* neither weakened its host to nor protected it from parasitism by *He. harmoniae*, and vice versa. A synergistic effect of co-infection by *He. harmoniae* and *Spiroplasma* in the same female resulted in significantly lower fecundity and hatchability rate. High hatchability rate was conditioned by high fecundity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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