

Fifty Shades of the Harlequin Ladybird and a Sexually Transmitted Fungus

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Subject Editor: Stefan Jaronski

Received 2 September 2019; Editorial decision 9 October 2019

Abstract

The ectoparasitic fungus *Hesperomyces virescens* was studied on its invasive host, the harlequin ladybird *Harmonia axyridis*, in the Czech Republic. A primary aim was to examine the relationship between fungal infection and elytral coloration of the ladybird. Furthermore, the role of host sex and mating status of females were analyzed. Beetles ($n = 1,102$) were sampled during autumn migration, and then sexed, weighed, and screened for infection. Females were dissected for detection of sperm in their spermathecae. Ladybirds were sorted according to color form and absorbance spectrophotometry was used to quantify carotenoid contents in their elytra. In individuals of the nonmelanic *succinea* form, the degree of melanization was measured using digital photographs and putative age groups were estimated based on background color of elytra. Sexual differences in infection patterns indicated transmission during copulation: males were infected mostly on elytra and venter, and females had infection almost exclusively on elytra. Mated females had higher infection rate than virgins. There was no influence of genetic color form on the fungal infection. Putative age groups (visual sorting to yellow, orange, and red) correlated with fungal infection. Infected individuals had elevated elytral carotenoid levels in comparison to uninfected individuals, which could be explained by host age. Infection-free *succinea* beetles were extensively melanized because they emerged later in the season at lower temperatures which induced melanization. Overall, we highlight that *H. axyridis* is a multivoltine species whose age, if not taken into account in ecophysiological studies, might present a considerable confounding factor.

Key words: *Harmonia axyridis*, *Hesperomyces virescens*, color pattern, carotenoids, melanism

The harlequin ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) is a beetle of Asiatic origin that has repeatedly been introduced in North America since 1916 to serve as biological control agent of agricultural pests (Roy et al. 2016). One invasive population from eastern United States (first observed in 1988) served as a bridgehead source for invasion to the European, South American, and African continents (Lombaert et al. 2010). Nowadays, *H. axyridis* has established populations in at least 59 countries outside its native range, including New Zealand (Camacho-Cervantes et al. 2017), and is considered as one of the most invasive alien species (IAS). Negative impacts of *H. axyridis* include the decline of native ladybird species (Brown and Roy 2017), toxicity to both humans and their pets (Goetz 2008, Stocks and Lindsey 2008), interference with wine production (Pickering et al. 2004), and acting as a household invader (Koch and Galvan 2008). *Harmonia axyridis* represents a well-established model species of global importance which is suitable for studies on invasion ecology and interactions with natural enemies (Haelewaters et al. 2017, Ceryngier et al. 2018).

Laboulbeniales are minute fungal ectoparasites that exhibit determinate growth and complete their entire life cycles on the integument of arthropod hosts (Haelewaters et al. 2012). In 2002, *Hesperomyces virescens* Thaxter (Ascomycota: Laboulbeniales) was found parasitizing *H. axyridis* in Ohio, United States (Garcés and Williams 2004). For a long time, *H. virescens* has been considered as eurytopic, infecting over 30 ladybird species worldwide (Haelewaters et al. 2017). However, a recent work provided evidence that *H. virescens* is in fact a cryptic complex of species, each with its own host (Haelewaters et al. 2018b). The thalli of *H. virescens* grow directly from sticky ascospores that adhere to the ladybird cuticle, forming a haustorial rhizoidal apparatus which penetrates into the host's hemocoel (for developmental morphology, see Weir and Beakes 1996). Direct contact during copulation is the main mode of transmission during the mating-feeding season as indicated by sex-dependent patterns of thalli distribution (Haelewaters et al. 2017). Although most Laboulbeniales are considered rather harmless, *H. virescens* causes increased mortality of ladybirds under laboratory conditions (Haelewaters et al. 2018c).

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In both North America and Europe, there was a time lag between the establishment of *H. axyridis* in the wild and the seeming acquisition of *H. virescens*. Even though this has been ascribed to putative host shifts from native ladybirds (Haelewaters et al. 2017), it is also possible that a *H. axyridis*-specific strain of the *H. virescens* cryptic species complex co-invaded with its host, as documented from Caucasus (Orlova-Bienkowskaja et al. 2018). The harlequin ladybird has since become the main host for *H. virescens*, expanding the distributional range of the fungus to northern (colder) localities. The ladybird exhibits some unique features that make it an especially suitable host for *H. virescens*: high promiscuity, multivoltinism with overlapping generations, and overwintering in mass aggregations (De Kesel 2011, Ceryngier and Twardowska 2013).

The ladybird *H. axyridis* is highly polymorphic in coloration (Tan and Li 1934, Tan 1946, Komai et al. 1950). Recent studies implied *pannier* (a transcription factor gene) to be the locus responsible for the major color pattern alleles (Ando & Niimi 2019). In Europe, the major alleles and color forms are *succinea*, *spectabilis*, *conspicua*, and *axyridis* (Brown et al. 2008, Michie et al. 2010). The *succinea* form is considered nonmelanic and predominates in most invasive populations worldwide. The extent of black pattern on the elytra of *f. succinea* ladybirds correlates negatively with temperature experienced during preimaginal development due to a phenomenon called thermal melanism (Michie et al. 2010). An elytron consists of a thin dorsal epidermis and thick ventral epidermis. Black melanins accumulate in the cuticle above the dorsal epidermis, while red pigments (carotenoids and pterins) accumulate in the thick ventral cuticle of *H. axyridis* (Ando and Niimi 2019). The major elytral carotenoids identified in *H. axyridis* are α -carotene, β -carotene, and lycopene (Bezzerides et al. 2007).

The harmonine alkaloid levels are negatively correlated with the extent of black pattern on the elytra of female *succinea* individuals (Bezzerides et al. 2007). Therefore, *H. virescens* infection was hypothesized to be associated with extensively melanized elytra, but no significant support for such hypothesis was found (Haelewaters et al. 2018a).

Because *H. axyridis* is multivoltine (Honek et al. 2018), changes in age structure of its populations over time definitely play a key role in *H. virescens* infection dynamics. Some researchers noted that host age might explain some of their findings and shed light on the *H. axyridis*–*H. virescens* association (Riddick and Schaefer 2005, Riddick 2006, Nalepa and Weir 2007), but no comprehensive analysis of age-dependent infection with *H. virescens* has been performed yet.

The work reported here had three objectives: 1) to examine the relationship between color polymorphism of the invasive ladybird *H. axyridis* and the infection with the fungal ectoparasite *H. virescens*. Coloration of the ladybirds was analyzed in terms of color pattern, carotenoid levels, and in *succinea* individuals also in terms of the degree of melanization. 2) To comprehensively demonstrate that infection with *H. virescens* is age-dependent. This was attained by sorting *succinea* individuals to putative age groups according to their background coloration. We hypothesized that redder ladybirds would have higher probability of being infected. 3) To revisit the influence of host sex and mating status on the infection with *H. virescens*.

Material and Methods

Sample Collection and Initial Processing

Adults of *H. axyridis* were collected during autumn flight to overwintering sites. They were landing on white walls of tall buildings in České Budějovice, Czech Republic (48°59'N, 14°27'E,

390 m in elevation). Sampling was conducted on four dates when the weather conditions were favorable for ladybird migration: 9 October ($n = 609$), 10 October ($n = 369$), 19 October ($n = 74$) and 10 November 2014 ($n = 50$). The ladybirds were placed in glass jars with crumpled filter paper (shelter) and a wet piece of cellulose (water source). They were subsequently maintained in a refrigerator at 5–10°C for a few days.

The fresh mass of the beetles was measured using an A&D GR-202 EC analytical balance to the nearest 0.1 mg. They were then sexed, sorted according to color pattern (*axyridis*, *conspicua*, *spectabilis*, or *succinea*), placed individually in 96-well microtiter plates and stored in a freezer at –20°C. Frozen individuals were then gradually processed.

Degree of Melanization

Beetles were photographed under standardized indoor lighting and background conditions using Nikon SMZ 1500 stereomicroscope with a build-in Lumenera INFINITY 2-5C digital camera. Structural body size—i.e., the length and width of both elytra (sensu Knapp and Nedvĕd 2013)—of all ladybirds was measured from the photographs using the QuickPHOTO CAMERA 3.0 software. In *succinea* individuals, the dimensions of two selected melanized spots (perisutural spots of the second and third transversal rows, Fig. 1, see Knapp and Nedvĕd 2013) were measured as well. The length and width of the spots were measured on the left elytron parallelly and perpendicularly to the elytral suture, respectively. Spot size was the product of spot length and spot width.

Approximated elytral area (Ae) was calculated as the product of the length and width of the elytra. Approximated spot area (As) was calculated by adding up spot sizes of the two melanized spots and multiplying the result by two. The degree of melanization was the percentage portion of elytra covered by these selected melanized spots, i.e., $100 \times (As/Ae)$.

Putative Age Groups

To assess whether there is a correlation between fungal infection and ladybird age, we sorted *succinea* individuals into three age groups according to their background elytral coloration: yellow, orange, and red (Fig. 1). Similar approach (suitable for prompt field analyses) is known from studies of the two-spotted ladybird *Adalia bipunctata* (Linnaeus) (Coleoptera: Coccinellidae) (Brakefield 1984, Welch et al. 2001), ants (Báthori et al. 2018), and Japanese/American populations of *H. axyridis* (Osawa and Nishida 1992, LaMana and Miller 1996).



Fig. 1. Typical yellow, orange, and red individuals of *H. axyridis* f. *succinea* categorized subjectively and representing putative age groups. Black arrow points to the thalli of *H. virescens*. Green triangles indicate the black spots used for melanization measurements, see Knapp and Nedvĕd (2013) for further details.

Screening for *H. virescens* Infection

Each ladybird was screened for the presence of mature *H. virescens* thalli under the stereomicroscope. The location of fungal thalli was mapped by dividing the insect body into six zones (Fig. 2). Note that unlike Nalepa & Weir (2007) we included prosternum in zone 1 (instead of zone 5) together with the head and pronotum. Zones with at least one mature thallus were recorded as infected, i.e., we did not count the number of thalli present in the zones.

Quantification of Carotenoids

Elytra of each ladybird were cut off using microscissors and placed in 1 ml of 96% ethanol in order to extract carotenoids. Ethanol samples with the elytra were then stored in a refrigerator for a few weeks. Subsequently, absorbance of each elytral sample was measured against blank ethanol sample at $\lambda = 450$ nm using a UVmini-1240 spectrophotometer. The absorbance of elytral carotenoid extracts was taken as a proxy for total carotenoid concentration and decadic-log-transformed prior to statistical analyses.

Determining the Mating Status

All female ladybirds were dissected under the stereomicroscope in Ringer solution, and their spermathecae were crushed on a microscopic slide to check for the presence of sperm.

Data Analysis

Fisher's exact tests on 2×2 contingency tables were used to compare infection rates between ladybird sexes for each zone separately (with α values adjusted according to the Bonferroni correction) and between mated and unmated females.

Comparison of infection rates between color forms was made using Fisher's exact test on a 4×2 contingency table.

We used a generalized linear model (GLM) with binomial error structure to test the effect of sex, carotenoid content (log-transformed absorbance), degree of melanization, mass, age group (red/orange/yellow), and size (approximated elytral area, i.e., the product of length and width of elytra) on the overall infection status (infection present/absent) of the f. *succinea* ladybirds. All two-way interactions were included in the model.

As the presence of infection in individual zones was not independent (Spearman's rank-correlation coefficients ranging between 0.45 and 0.72 for ventral zones), the total number of infected zones per ladybird could not be used as a response variable in a GLM with Poisson or Quasi-Poisson error structure. Thus, to analyze infection severity, we used infected *succinea* individuals only ($n = 252$). We classified ladybirds with one infected zone as lightly infected and those with two or more infected zones as heavily infected. We then fitted a GLM with binomial error structure to assess the effect of sex, carotenoid content, degree of melanization, mass, age group,

and size on the infection severity (infection heavy/light). All two-way interactions were included in the model. The only three yellow individuals eligible were re-classified as orange (for the purpose of this analysis only) to balance the age group factor variable and enable interactions with other explanatory variables. To avoid issues with multicollinearity, we standardized all continuous variables in both GLM models described.

We found minimum adequate models by the backward stepwise elimination approach. To compare models containing factors of interest with models omitting these factors, χ^2 likelihood ratio tests were used. We used the *dropterm* function in the *MASS* package for model comparisons. The relevance of minimum adequate models was checked by computing the Akaike Information Criterion (AIC) values for the whole model space and examining variable importance calculated from AIC weights. All possible submodels were explored using the *dredge* function in the *MuMIn* package. To aid the interpretation of any significant interaction terms in the models, the variable effects were explored using the *effects* package (Fox 2003). All analyses were performed using R (v. 3.5.1; R Core Team 2019).

Results

Sexual Differences in Infection

Out of 1,102 migrating ladybirds sampled, 287 individuals bore thalli of *H. virescens* (prevalence = 26 %). Regarding the location of infection (Fig. 3), females were most often infected on the posterior half of elytra (zone 3). In addition to posterior elytra, males were also infected in ventral zones (4, 5, and 6). Inter-sexual differences were significant for ventral zones only (Fisher's exact tests: Bonferroni-adjusted $\alpha = 0.0083$, $P < 0.0001$ for each of the three zones).

Mating Status of Females

Overall 259 out of 523 females were mated (49.5 %). The infection rate was significantly dependent on mating status (Fisher's exact test: $P < 0.0001$); with more mated (35.9%) than unmated (10.6%) females being infected. Note that if reported conversely (i.e., mated percentage of infected females instead of infection rates among mated and unmated females), the results would be 76.9% of infected females were mated, while 7.0% of uninfected females were mated.

In both mated and unmated females, infection was located most often on the posterior elytra (zone 3). There were 32.0 and 9.8% of mated and unmated females, respectively, infected on the posterior elytra.

Color Forms

No relationship between infection rate and color form was found (Fisher's exact test: $P = 0.66$). Overall, infection with *H. virescens* occurred in 33% of *axyridis* individuals ($n = 3$), 24% of *conspicua* individuals ($n = 29$), 31% of *spectabilis* individuals ($n = 88$), and 26% of *succinea* individuals ($n = 982$).

Modeling Infection Status

The effect of carotenoid content on infection status varied with mass (interaction: $\chi^2 = 4.03$, $df = 1$, $P = 0.045$). Heavy ladybirds were more likely to be infected with increasing carotenoid content. Lighter ladybirds were less likely to be infected with increasing carotenoid content. The relationship is visualized in Fig. 4. Overall, carotenoid content increased with increasing mass, but this effect was reduced for uninfected individuals (which were generally less carotenoid-rich than infected individuals). The minimum adequate model comprised no other interactions.

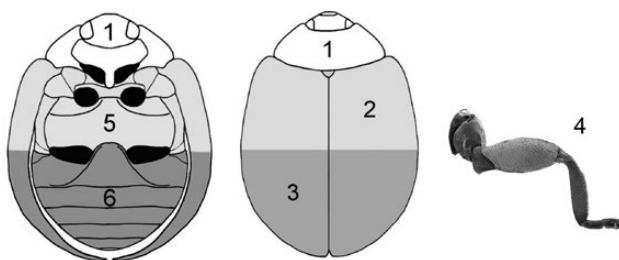


Fig. 2. Six zones delimited on the ladybird body in order to score the extent of *H. virescens* infection. Modified after Nalepa and Weir (2007).

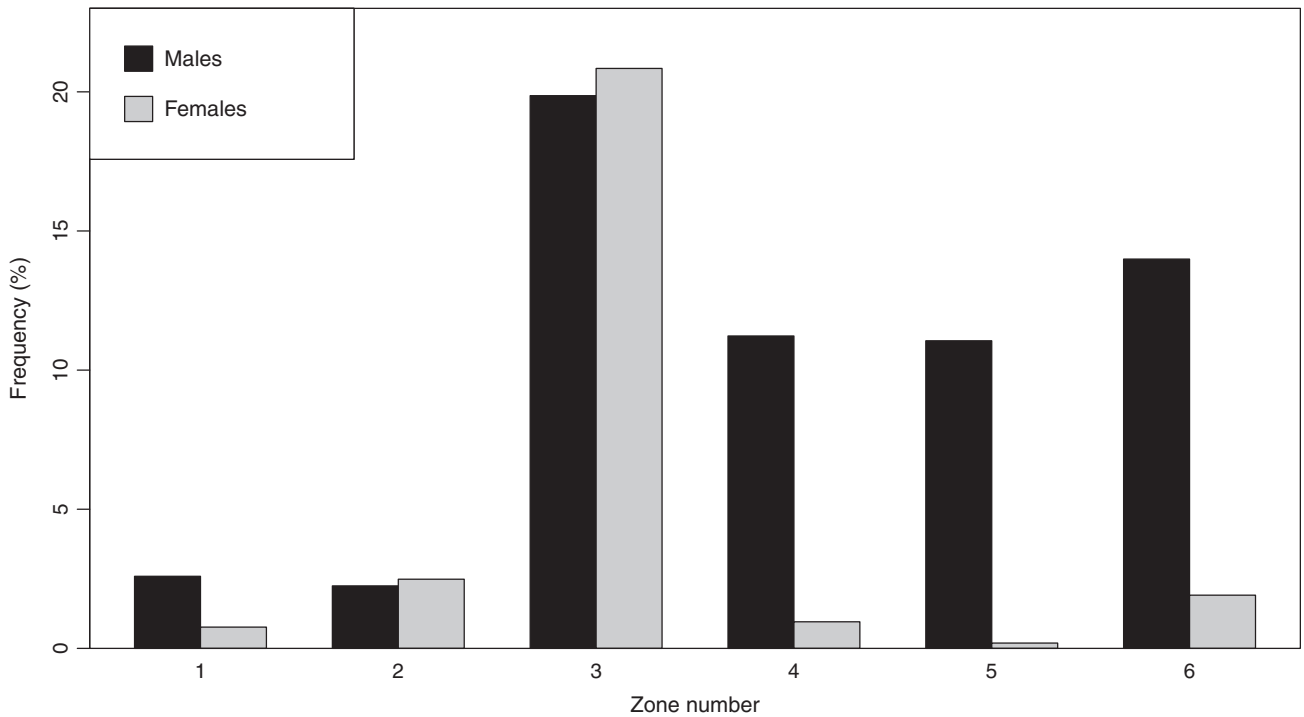


Fig. 3. Frequency of infection of individual zones in males ($n = 579$) and females ($n = 523$).

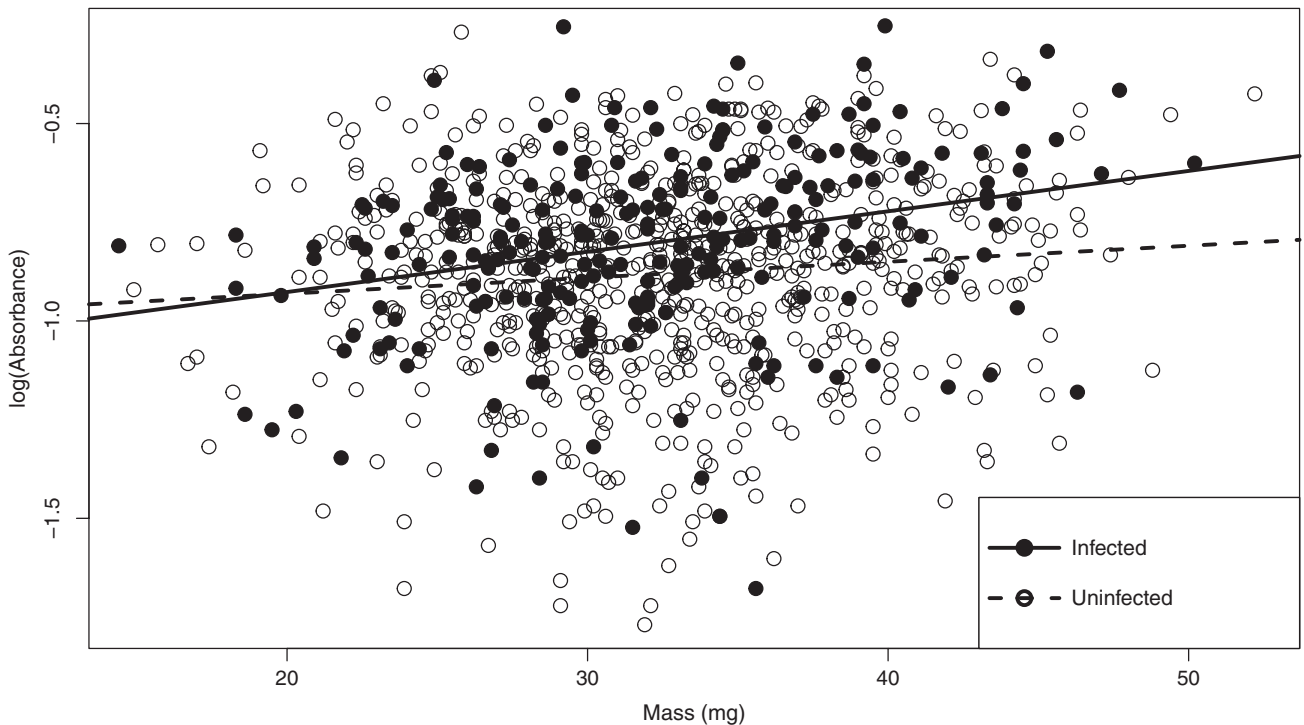


Fig. 4. The relationship between infection status, elytral carotenoid content (log-transformed absorbance), and fresh ladybird mass. The slopes and intercepts for the lines of best fit were obtained from a general linear model: $\log(\text{Absorbance}) \sim \text{infection}:\text{mass}$, and are shown to guide the eye. Infected individuals had increased elytral carotenoid contents and this effect was more pronounced with increasing mass.

Red ladybirds were more likely to be infected than orange or yellow ladybirds ($\chi^2 = 42.62$, $df = 2$, $P < 0.0001$). There were 43.8% of red individuals infected ($n = 267$), 19.0 % of orange individuals infected ($n = 696$), and 16 % of yellow individuals ($n = 19$) infected with *H. virescens*.

Ladybirds with increasing degree of melanization were less likely to be infected ($\chi^2 = 22.33$, $df = 1$, $P < 0.0001$). In other words, infected individuals had smaller percentage of elytra melanized (8.20 ± 0.32 %) than uninfected individuals (10.29 ± 0.19 %), mean \pm standard error of the mean.

Ladybird sex ($\chi^2 = 0.10$, $df = 1$, $P = 0.75$) and size ($\chi^2 = 3.08$, $df = 1$, $P = 0.08$) were not significant predictors of infection status.

AIC approach to the starting GLM confirmed the degree of melanization and age group as highly important variables (i.e., present in all submodels with any weight). The interaction between carotenoid content and mass was the only moderately important interaction (variable importance = 0.61), all remaining two-way interactions were not important (variable importance < 0.50).

Modeling Infection Severity

Infected males were more likely to have fungal thalli in more than one zone (= heavily infected, see Fig. 2) than infected females ($\chi^2 = 55.06$, $df = 1$, $P < 0.0001$). There were 55.9% of infected males ($n = 145$) with more than one zone occupied versus 13.1% of infected females ($n = 107$) with more than one zone occupied.

The effect of age group on infection severity was significant ($\chi^2 = 7.41$, $df = 1$, $P = 0.007$) with red infected ladybirds being most likely to bear fungal thalli in more than one zone. There were 44.4% of infected red individuals ($n = 117$) with *H. virescens* in more than one zone and 31.9% of infected orange individuals ($n = 135$) with *H. virescens* in more than one zone.

The minimum adequate model included no interactions. The effect of the following variables on infection severity was not significant: carotenoid content ($\chi^2 = 0.00$, $df = 1$, $P = 1.00$), degree of melanization ($\chi^2 = 0.03$, $df = 1$, $P = 0.87$), mass ($\chi^2 = 2.25$, $df = 1$, $P = 0.13$), and size ($\chi^2 = 1.98$, $df = 1$, $P = 0.16$).

AIC approach indicated that both sex and age group were highly important variables (variable importance = 1.00, 0.95, respectively).

Discussion

Sexual Differences in Infection

The infection was most prevalent on the dorsoposterior of females and on the dorsoposterior and venter of males. This distribution neatly fits the sexual transmission scenario (Welch et al. 2001) except for the high fungal incidence on the posterior elytra of males. Same-sex mounting might be responsible for this peculiar location of thalli in males (Nalepa and Weir 2007). Mature males are very active and mount almost anything of the right size and shape without regard to species or sex (Obata 1987). The finding that males were infected more severely than females is in accordance with literature (Haelewaters et al. 2018a).

Mating Status of Females

Higher infection rate of mated females in comparison to unmated females confirms that *H. virescens* infection is transmitted during copulation. In contrast to Nalepa and Weir (2007), we found the infection to be better correlated with the presence of sperm in the spermathecae. In North Carolina, 37.7 and 66.7% of infected females and 24.2 and 34.4% of uninfected females in a mountain and piedmont site, respectively, were mated during the 2003 autumn flight (Nalepa and Weir 2007). In this study, 76.9 and 7.0% of infected and uninfected females, respectively, were mated.

Unsuccessful copulatory attempts (Obata 1988) might be responsible for the infection of virgin females (10.6% of all virgins were infected in this study). The infection pattern of unmated females was similar to that of mated ones with thalli being most often on their posterior elytra (data not shown).

Color Forms

There was no influence of ladybird color form on the infection rate. This is interesting because various *H. axyridis* color forms are

known to exhibit many properties (mostly mediated by thermal physiology) that could make them diversely suited as hosts of *H. virescens*. These are, for example, mating frequency, winter survival, and ability to spread during the invasion process (Osawa and Nishida 1992, Su et al. 2009, Awad et al. 2015, Purse et al. 2015). Moreover, *H. axyridis* color forms seem not to exhibit intraspecific variation in immunity against *H. virescens*.

Coloration Parameters: Carotenoids

Elevated carotenoid levels in the elytra of *H. axyridis* individuals infected with *H. virescens* reflect either i) host age differences or ii) upregulated immune response of infected ladybirds. These two explanations are not mutually exclusive. The difference in carotenoid contents was more pronounced in heavier ladybirds.

- (i) Older *H. axyridis* individuals had higher probability of encountering the infection with *H. virescens* and they had more time to accumulate carotenoids in elytra, hence the observed correlation.

In some studies, generations of multivoltine ladybirds were differentiated according to pale and soft elytra indicating recent adult emergence (Osawa and Nishida 1992, LaMana and Miller 1996, Ceryngier and Romanowski 2017). Carotenoid content in the elytra of *H. axyridis* is correlated to the visual red color intensity (Bezzarides et al. 2007).

Most animals must acquire carotenoids from their diet, only some phytophagous insects and mites can synthesize them de novo due to horizontal gene transfer from fungi or bacteria (aphids: Moran and Jarvik 2010, whiteflies: Sloan and Moran 2012, mites: Altincicek et al. 2012). We, therefore, assume that *H. axyridis* must obtain carotenoids from its diet as most animals do. This is supported by pale color of individuals reared on artificial food based on chicken liver, yeast extract, milk powder, wheat germ oil, and honey (Nedvĕd, personal observation). It seems well confirmed that carotenoids are accumulated as a function of the ladybird's age (Nedvĕd et al. 2019), but other factors likely modulate this trend. Namely, carotenoids play an important role in the immune system (McGraw 2005).

- (ii) In vertebrates carotenoids are well known for their antioxidant and immunostimulatory functions (Svensson and Wong 2011). In invertebrates the role of carotenoids as antioxidants has been studied to a much lesser extent (Felton and Summers 1995). Cornet et al. (2007) showed that the use of the prophenoloxidase system strongly positively correlated with carotenoid concentration in hemolymph of the crustacean *Gammarus pulex* (Linnaeus) (Crustacea: Amphipoda). The causality of this relationship was confirmed because dietary supplementation with carotenoids enhanced phenoloxidase activity without inducing additional self-harming in the crustacean (Babin et al. 2010). Moreover, a recent study demonstrated that redder and darker clypeal membranes in *Nicrophorus pustulatus* Herschel (Coleoptera: Silphidae) beetles are associated with an increased induced immune response (Wormington and Luttbeg 2018).

Regarding Laboulbeniales in relation to host immunity, association between the invasive ant *Lasius neglectus* Van Loon, Boomsma & Andr sfalvy (Hymenoptera: Formicidae) and its ectoparasite *Laboulbenia formicarum* Thaxter (Ascomycota: Laboulbeniales) was studied by Konrad et al. (2015). They found that ants with high *Laboulbenia* levels showed elevated expression of immune genes relevant for wound repair and antifungal responses (β -1,3-glucan binding protein, Prophenoloxidase) compared with ants carrying low *Laboulbenia* levels. The fungus also mediated

prophylactic protection against the entomopathogen *Metarhizium brunneum* Petch (Ascomycota: Hypocreales) (Konrad et al. 2015). Whether similar beneficial association between an invasive insect and laboulbenialen fungus contributed to the spread of the *H. axyridis*–*H. virescens* association remains a question.

Sometimes, small areas of darkened elytral cuticle from which *H. virescens* thalli grow can be observed in heavily infected individuals (Fiedler and Nedvĕd, personal observation). Carotenoids might be scavenging free radicals produced by the activity of phenoloxidase. Concordant with the supposed role carotenoids may play in the ladybird's immune system is a finding that β -carotene-amended artificial diet increases larval survival of *H. axyridis* (Sun et al. 2018). We note that the *H. axyridis*–*H. virescens* association is a unique system to study the role of carotenoids in immune response with potential implications for invasion ecology and pest management in general.

Coloration Parameters: melanization

We found that *f. succinea* individuals with increased proportion of elytra melanized were more likely to be infected with *H. virescens*. This is a strong evidence for age-related infection development. The nonmelanic form *succinea* dramatically increases its degree of melanization at cold temperatures, a phenomenon called thermal melanism (Michie et al. 2010). There was a significant increase in the degree of melanization over the course of autumn in our study (data not shown). This means that adults of young generation, that emerged later in the season at lower temperatures, arrived at the overwintering site on late sampling dates. Lower temperatures induced extensive melanization which we then found to be associated with infection-free individuals as they had lower chance of encountering *H. virescens* during their shorter adult lifetime.

Haelewaters et al. (2018a) hypothesized that the infection with *H. virescens* would be associated with extensive elytral melanization of *succinea* individuals because black coloration should correlate negatively with the the harmonine alkaloid levels (Bezzerides et al. 2007). They found no significant support for this prediction and our data show exactly the contrary to what they had predicted. Bezzerides et al. (2007) studied the relationship between harmonine contents and coloration of *H. axyridis f. succinea* but they omitted thermal melanism and explained their findings only in terms of aposematic signaling. They sampled *H. axyridis* from the wild during winter so their sample likely comprised beetles of various generations. Here we argue that variable age (or variable temperatures experienced during preimaginal development) of the ladybirds might have accounted for elevated alkaloid levels in their study. Individuals with high alkaloid levels had small extent of black coloration on their elytra but were also older than heavily melanized beetles. *H. axyridis* is, therefore, a multivoltine species whose age, if not taken into account during ecophysiological studies, might present a considerable confounding factor.

Putative Age Groups

Visually estimated age categories of *succinea* individuals showed correlation with infection rate and severity as predicted. Red individuals were more likely to be infected than orange and yellow ones. Infection of red individuals was also more severe than that of orange and yellow beetles. The red hue of *H. axyridis f. succinea* elytra is caused by a range of chemicals dominated by carotenoids and pterins (Bezzerides et al. 2007). These are accumulated as a function of ladybird age (Nedvĕd et al. 2019). Our results show that age of the harlequin ladybirds can be estimated subjectively based on their elytra coloration. This is useful for scientists who aim to sample *H. axyridis* individuals infected with *H. virescens*. The best time to sample

parasitized ladybirds is early autumn when older generation migrates to overwintering sites. Moreover, selecting intensely red individuals will yield high fungus intensities for laboratory experiments.

Although social transmission of the fungus may be important during overwintering in large tight clusters, the pattern of infection in our samples (sexual differences, the distribution of thalli on ladybird bodies, the importance of age, and mating status) clearly confirms *H. virescens* as a sexually transmitted disease.

Summary of Major Findings

We investigated the influence of ladybird coloration on the *H. virescens* ectoparasite. Elytral carotenoid levels positively correlated with infection status. This raises interesting questions with regard to the effects of ladybird age and immunity. Further manipulative experiments will be needed to elucidate the role of carotenoids in the immunity of ladybirds. The hypothesis that individuals visually sorted as red (older) would be more likely to bear fungal thalli has been confirmed. We found that increased elytral melanization of *f. succinea* ladybirds is associated with low likelihood of infection. Thermal ecophysiology of *H. axyridis* can explain this pattern very neatly. Ladybirds with increased degree of melanization emerged at lower temperatures later in the season. They were, therefore, younger and had fewer chances of contracting fungal infection.

Acknowledgments

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors. We would like to thank Danny Haelewaters for his valuable comments. We thank two anonymous reviewers for improving our manuscript. The first author acknowledges Andrea Manica for his insightful comments and advice on statistical analyses.

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