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Article

Influence of Elytral Colour Pattern, Size, and Sex of *Harmonia axyridis* (Coleoptera, Coccinellidae) on Parasite Prevalence and Intensity of *Hesperomyces virescens* (Ascomycota, Laboulbeniales)

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Abstract: *Harmonia axyridis* is an invasive ladybird (Coleoptera, Coccinellidae) with the potential to outcompete native ladybird species in its invasive distribution area. It was introduced as a biological control agent in many countries but has also spread unintentionally in many others. *Hesperomyces virescens* (Ascomycota, Laboulbeniales) is a minute (200–400 µm in size) biotrophic fungus that infects over 30 species of ladybirds. The aim of this study was to evaluate whether elytral colour pattern, size, and sex of *Ha. axyridis* affect infection by *H. virescens*. Colouration in *Ha. axyridis* has been linked to the presence of an antimicrobial alkaloid (harmonine). In fall 2016, we collected 763 *Ha. axyridis* individuals in Cambridge, Massachusetts, of which 119 (16%) bore *H. virescens* fruiting bodies. We analysed 160 individuals concerning prevalence and intensity of infection by *H. virescens*. Elytral sizes and colouration patterns were quantified using digital photography and analytical methods. Smaller ladybirds had a higher prevalence and higher intensity of parasitism. Additionally, male ladybirds bore more thalli compared to female ladybirds. Elytral colour patterns had an effect on neither prevalence nor intensity of infection by Laboulbeniales in our dataset. This suggests that development of Laboulbeniales may be unaffected by certain insect alkaloids.

Keywords: biotrophic interactions; invasive species; colour polymorphism; harlequin ladybird; harmonine

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1. Introduction

The harlequin ladybird *Harmonia axyridis* (Coccinellidae, Coleoptera) is arguably one of the best studied and well-known examples of invasive insect species. Native to Eastern Asia, it was intentionally introduced as a biological control agent of aphids and scale insects first in the USA and later in various European countries. In recent years, however, it has also spread unintentionally in Northern and Southern America, Europe, and parts of Asia outside of its native range [1,2]. In Africa, *Ha. axyridis* was introduced intentionally in South Africa (unsuccessful), Egypt, and Tunisia [1]. The recent invasion in South Africa is the result of unintentional introduction [1], whereas the (small) populations in Kenya and Tanzania may represent transient introductions [3,4]. The global invasion of *Ha. axyridis* happened quickly and inspired different facets of research dealing with this species. *Harmonia axyridis* is a major concern, since it causes the displacement of native ladybird species, threatening native ecosystem services [5], and commercial losses in the wine industry [6]. Therefore, efforts are made to find ways to control invasive populations of *Ha. axyridis*, justifying intensive research regarding its natural enemies.

Our group is particularly interested in one of these enemies – *Hesperomyces virescens* (Ascomycota, Laboulbeniales). *Hesperomyces virescens* is a minute (200–400 µm in length) biotrophic fungus that infects over 30 species of ladybirds belonging to 20 genera [7,8]. In recent years, parasite prevalences have increased on *Ha. axyridis*, because this ladybird combines a number of behavioural and life history features that are beneficial for the spread and acquisition of the fungus. Overwintering in large aggregations and a highly promiscuous lifestyle (including males copulating with males) are the most important traits because they allow for many intra- and inter-generational contacts. Like all other Laboulbeniales, *H. virescens* is transmitted nearly exclusively during direct contact between host individuals, especially mating [6, 7]. Although delayed in occurrence after establishment of *Ha. axyridis*, *H. virescens* has been reported from *Ha. axyridis* in most areas of its occurrence. Discovered in the USA in 2002 [11], the *H. virescens*–*Ha. axyridis* combination was later observed in various European and South American countries and South Africa [7,12,13]. In addition, two infected specimens of *Ha. axyridis* collected in China in the 1930s were retrieved during museum collection studies [14]. Due to the charismatic character of its ladybird hosts, their importance in our ecosystems, and the status of its most common host *Ha. axyridis*, *H. virescens* has become one of the best studied species of Laboulbeniales. Seasonal variation of *H. virescens* prevalence was explored in a few publications [5, 12] as well intra- and interspecific transmission successes [16] and negative effect on its hosts [9,17,18].

In this study we explored the potential link between of colour polymorphism of *Ha. axyridis* and the prevalence and intensity of *H. virescens* infection. *Harmonia axyridis* is highly polymorphic in colour patterns; this polymorphism is controlled by one locus with 15 alleles [19]. The bright, multi-spotted *forma succinea* is usually the most abundant phenotype [15, 16]. However, the proportion of individuals with different phenotypes varies across seasons, mediated by a balance of climate factors, pollution, non-random mating behaviours, and sexual selection [22–27]. Moreover, ladybirds of different phenotypes are known to have distinct invasion patterns [28]. Interestingly, the degree of melanisation is known to vary depending on environmental conditions, even within the same phenotype [19]. In *forma succinea*, the proportions of black to bright areas (ranging from entirely bright to nearly entirely black) are dependent mostly on temperatures during larval and pupal development. Even though melanic forms of *Ha. axyridis* (*f. axyridis*, *f. conspicua*, *f. spectabilis*) are better adapted to cold conditions [20, 21], black colouration is negatively correlated with total alkaloid content, at least in females [29]. Insect alkaloids serve as deterrents against predators but they are also considered as non-specific defences against pathogens [30]. Here, we aimed to investigate whether colour patterns, and thus indirectly alkaloid contents, have an influence on parasitism by *H. virescens*. We hypothesised that both the parasite prevalence and intensity of infection by *H. virescens* are elevated for ladybirds with higher melanic area on their elytra (i.e. with increased number of spots and/or increased spot size).

2. Materials and Methods

A total of 763 specimens of *Harmonia axyridis* ladybirds of *forma succinea* were collected from the South and West walls of William James Hall, Cambridge, Massachusetts in October–November 2016. All individuals were sexed and screened for infection with *H. virescens* in the laboratory using an Olympus SZX9 stereomicroscope (Waltham, Massachusetts) at 50×. Of these ladybirds we used a balanced dataset of 160 individuals, 80 males (40 infected, 40 uninfected) and 80 females (40 infected, 40 uninfected), for further analyses. For each individual, we measured the area of the left elytron and calculated the percentage of the elytral area covered by black spots. For this purpose, we made images of all 160 left elytra using an Olympus XC50 camera and cellSens Standard 1.14 software (Olympus). All images are available for download from the Figshare online repository [31]. To develop an automated method measuring total elytral area and elytral area covered by spots as well as counting number of spots/left elytron, we designed a macro for the ImageJ platform [32] and coded the script using IJM programming language on Fiji image processing software [33]. The most relevant image processing steps are detailed below.

For each left elytron image (Figures 1A–C), we manually drew the RIO (= Region of Interest) using the Polygon tool in ImageJ (Figure 1D). The macro starts by asking the user to select three folders: a folder with all raw images of left elytra (RGB format, TIFF files), a folder with all left elytron ROIs (_roi.roi files) and a folder for results. Through modification of each RGB image (Colour Deconvolution plugin), the spot regions are detected (Figure 1E) and drawn in binary fashion, as white ROIs on a black background (Figure 1F). The macro then counts the number of detected spots and measures the area of the left elytron ROI and of each spot ROI. The number of spots and measurements are saved in an Excel file in the result folder. The first measurement is the area of the total left elytron ROI, the other measurements (2, 3, 4, etc.) are the areas of the spot ROIs. A single measurement in the resulting Excel file indicates absence of spots. The macro is available for download from Figshare [31].

All statistical analyses were performed in R [34]. We used general linear models to investigate whether the colour pattern has an influence on prevalence and intensity of parasitism. We used the elytral area and the spot percentage as explaining variables for, first parasite prevalence, and second thallus count as response variables. Because the colour pattern presented a significant gender bias, we used the interaction of spot percentage and sex in all our models. We further included the variable sex in our model addressing the thalli count. All continuous variables were standardized to control for differences in magnitude between variables.

We used a likelihood ratio test to compare our candidate model for each, prevalence and intensity, containing elytral area and spot percentage as explaining variables ($Mod1_{prev}$, $Mod1_{int}$) with the respective Null model ($Mod0_{prev}$, $Mod0_{int}$) and, furthermore, calculated pseudo R2-values to evaluate model fit with the help of R package *sjstats*, using the function r2() [35].

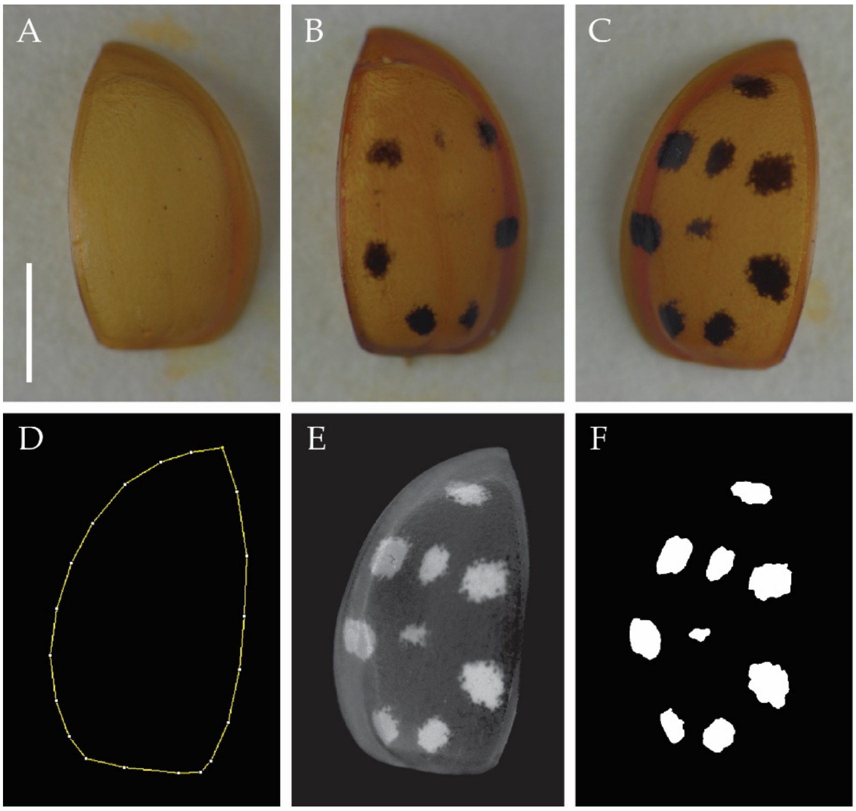

Figure 1. (A–C) Examples of different elytral sizes and colouration patterns in *Harmonia axyridis forma succinea*. (D–F) Different image processing steps for the raw image shown in C. (D) Left elytron ROI drawn manually using the Polygon tool. (E) Modified image after colour deconvolution for easy detection of spots. (F) Spot ROIs. Scale bar = 2 cm.

3. Results

Of the 763 sampled *Ha. axyridis*, 119 individuals were infected with *H. virescens* (parasite prevalence = 16%). We did not find any sex biases in the prevalence, with males (63 out of 423, 15%) being equally likely to be infected as females (56 out of 340, 16%) (Chi-Squared-Test, X^2=0.36, *p*=0.55). During data exploration we detected a significant sex bias in the colour pattern, with males (mean=10.6%) having less area of their elytra covered by black spots than females (mean=20.5%) (Kruskal-Wallis test, X^2=23.4, *p*<0.001). Interestingly, we did not find differences in total elytral area between males and females (t-test, t=0.66, df=158, *p*=0.51).

To determine whether colour pattern is linked to prevalence and intensity of parasitism with *H. virescens*, we used general linear models. Addressing the parasite prevalence, we included 160 individual beetles, 80 males and 80 females, in our model ($Mod1_{prev}$), which was significantly better than the Null model ($Mod0_{prev}$) (Chi-squared-Test, X^2=10.4, *p*=0.03). Although we did not find a link between spot area and parasite prevalence, total elytral area had a strong negative effect, indicating a higher parasite prevalence for smaller elytra (Table 1, Figure 2A). The overall model fit however was quite low (Nagelkerke's R-squared=0.08), suggesting further variables not included in our model also have an effect on prevalence.

Table 1. Results for model addressing prevalence of infection of *Ha. axyridis* with *H. virescens*.

Explanatory variable	Estimate	Std. Error	z value	*p* value	
(Intercept)	-0.0486	0.1776	-0.2740	0.7843	
Elytral Area	-0.4896	0.1700	-2.8790	0.0040	*
Spot Percentage : Sex f	-0.0061	0.2313	-0.0260	0.9791	
Spot Percentage : Sex m	-0.2642	0.2558	-1.0330	0.3016	

*Significantly better than $Mod0_{prev}$.

Intensity of parasitism was measured by counting the number of thalli on each individual of the 80 infected beetles (40 females and 40 males) and the resulting model ($Mod1_{int}$) was significantly better than the Null model ($Mod0_{int}$) (Chi-squared=23.2, *p*<0.001). We found a strong positive effect of the sex of the beetle, with males having significantly more thalli compared to female beetles. The elytral area again showed a significant negative effect, indicating more thalli on smaller elytra (Table 2, Figure 2B). Although spot area had no significant effect on intensity of parasitism, we noticed a marginally significant trend for male beetles with higher spot percentage presenting higher numbers of thalli (*p*=0.0513, Table 2). The overall model fit was medium (Nagelkerke's R-squared=0.25), suggesting further variables influencing the intensity of parasitism.

Table 2. Results for model addressing intensity of infection of *Ha. axyridis* with *H. virescens*.

Explanatory variable	Estimate	Std. error	z value	*p* value	
(Intercept)	3.0004	0.0385	77.9380	<2e-16	*
Sex m	0.2028	0.0524	3.8690	0.0001	*
Elytral Area	-0.0534	0.0240	-2.2230	0.0262	*
Spot Percentage : Sex f	0.0165	0.0357	0.4620	0.6438	
Spot Percentage : Sex m	0.0745	0.0383	1.9490	0.0513	(*)

*Significantly better than $Mod0_{int}$; $^{(*)}$marginally significant.

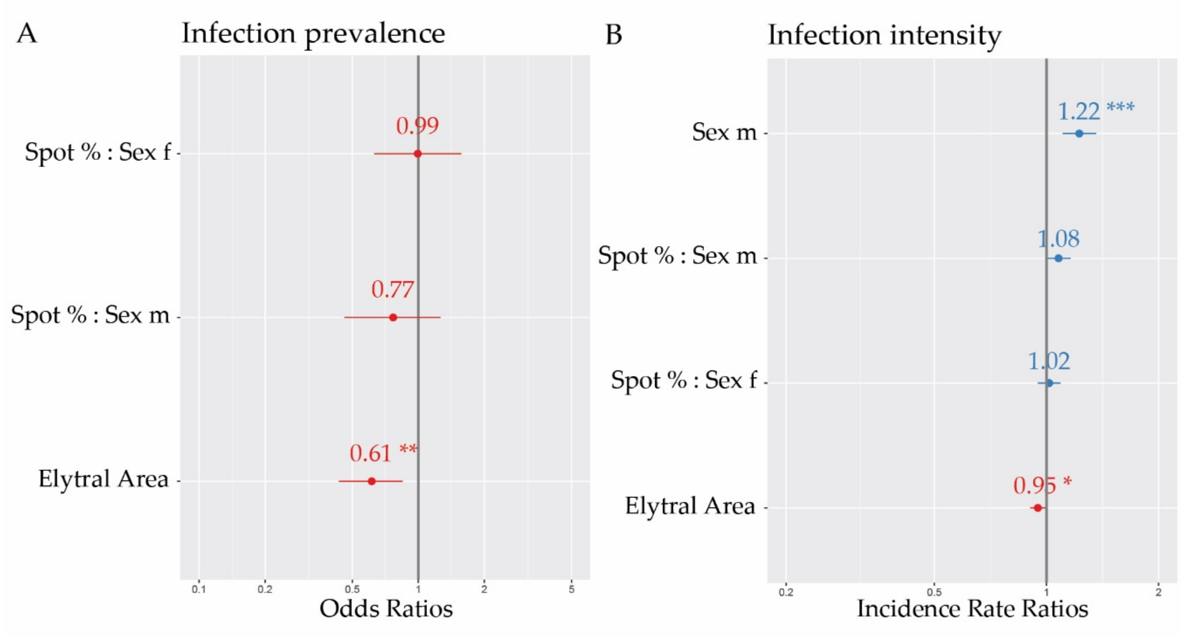

Figure 2. Forest plots representing the results of our modelling approach, showing in (**A**) a significant negative effect of total elytral area on the prevalence of infection of *Ha. axyridis* with *H. virescens* and in (**B**) a significantly higher intensity of parasitism in males compared to females as well as a significant negative effect of total elytral area.

4. Discussion

During this study we collected 763 specimens of the harlequin ladybird *Ha. axyridis* of which 119 (16%) were infected with *H. virescens.* Parasite prevalence did not differ significantly between sexes in our studied population. Whereas previous studies all observed significant differences in parasite prevalence over space and time, there is a trend for male ladybirds to have higher parasite prevalences compared to females [15,36]. This trend is significant in most studies, but in North Carolina there was only significance for one sample (out of four) [9]. The main reason proposed for this outcome is variation in number of contacts of females with infected males prior to arriving at aggregation sites during Fall [*sensu* 37]. Concordant with previous studies, we found higher intensity of infection in male ladybirds [6,26]. This has been explained by male mating behaviour resulting in a greater chance of contact with other infected individuals. Particularly indiscriminative mating of males with either sex is considered as the major cause for the observed infection patterns [9].

Our hypothesis that the colour pattern of *Ha. axyridis* influences infection with *H. virescens* was not supported. The degree of elytral melanisation did not have a significant negative impact on either prevalence or intensity of parasitism. We even found a slight trend to higher intensity of parasitism in more melanic males. This may indicate that insect alkaloids do not affect the pathogenicity of the fungus. However, we did not directly measure alkaloid contents and the correlation between blackish colouration and concentration of harmonine was only found significant for females [29]. Recently, another type of immune defence was indicated as a potential advantageous innovation of *Ha. axyridis*. Antimicrobial peptides were shown to be potent inducible defences against both bacteria and fungi [38]. Two antibacterial c-type lysosymes augment the two components (harmonine and antimicrobial peptides) of the immune response of *Ha. axyridis* [39]. Because little is known about the infection mechanisms of Laboulbeniales, we do not know what types of immune defence they are prone to. Nevertheless, the *Ha. axyridis*–*H. virescens* system may be the perfect model to study these questions.

To our knowledge, this is the first study linking size of ladybirds to infection by *H. virescens*. We found that, independent of sex, ladybirds with smaller elytral area (and thus smaller general size) were infected more often and with higher intensity. We hypothesize that this might be linked to differences in immune response, physical features of the cuticle, or activity between larger and smaller ladybirds. First, small size may be indicative of a weaker immune defence against parasites.

Adult body size reflects larval food supply [40]. Indeed, what larval stages receive as nutrition significantly affects the size achieved by adults [41]. Second, it is possible that smaller individuals are more prone to infection of *H. virescens* because of a thinner cuticle that is easier to penetrate [42]. At least for ants (Hymenoptera, Formicidae), a strong correlation between cuticle thickness and body size has been found [43]. With regard to Laboulbeniales infections, larger species of insects with thicker cuticles carry thalli less often than their smaller relatives [29]. Interestingly, species with thick cuticles are infected primarily on their most vulnerable body parts, that is, those areas where the cuticle is thinnest [30]. A prime example are the antennae of cockroaches (Blattodae), where the majority thalli of *Herpomyces* spp. seem to be located [42,44].

Our third hypothesis states that smaller ladybirds may be more active during mating, implying more physical contact for transmission of ascospores. Body size indeed is an important character that has been implicated in affecting competitive capacity, mating success, survival, and other life history traits of organisms [45,46]. During a study in the native range of *Ha. axyridis*, mating males of the spring generation were significantly larger than non-mating ones [22]. Later, again using *Ha. axyridis* specimens collected in the native range (Japan), mating non-melanic males were found to be significantly larger than non-mating ones [47]. In another study focusing on thermal properties of *Adalia bipunctata* [48], the effect of body size on walking speed was evaluated. The authors found no significant differences, except at 3 °C; at that temperature larger ladybirds tended to walk faster than smaller ones. With regard to reproductive success, the maximum number of eggs laid per day is larger for *Coccinella septempunctata* compared to *Propylea quatuordecimpunctata*, a much smaller species [49]. It seems most studies agree that large body size may be beneficial for reproductive success. In addition, one study was able to positively link large size to activity (walking speed). Based on the available data from previous studies, the hypothesis that smaller ladybirds may have a higher activity during mating cannot be supported Focused field and experimental studies are needed to elucidate which factors truly have an impact on this insect–fungus interaction.

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Author Contributions: D.H. initiated the research project. D.H. and M.G. sampled ladybirds. M.G. screened ladybirds and made images of left elytra. D.H. processed images in ImageJ/Fiji. T.H. performed statistical analyses. All authors contributed to writing and editing the manuscript.

Conflicts of Interest: The authors declare that there is no conflict of interest.

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