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Fungal ectoparasites increase winter mortality of ladybird hosts despite limited effects on their immune system

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Winter represents a challenging period for insects inhabiting temperate regions. A plethora of studies have investigated how environmental conditions such as temperature affect insect overwintering success. However, only a few studies have focused on biotic factors and the mechanisms affecting the overwintering performance of insects. Here, we investigated the effects of the parasitic fungus *Hesperomyces virescens* on the overwintering performance and immune system functioning of the invasive ladybird *Harmonia axyridis*. Winter survival was significantly lower for infected than for uninfected ladybirds. Body mass loss during overwintering tends to be higher for infected individuals compared to uninfected ones and for larger ladybirds. In addition, parasitic infection reduced post-winter longevity without food in male but not female ladybirds. Total haemocyte and protein concentration as well as antimicrobial activity against *Escherichia coli* significantly decreased during ladybird overwintering. However, haemolymph parameters were only poorly affected by *Hesperomyces* infection, with the exception of antimicrobial activity against *E. coli* that tended to be higher in infected ladybirds. Interestingly, none of the pre-winter haemolymph parameters were good predictors of ladybird winter survival. Overall, our results indicate that energy exhaustion unrelated to immune system challenge is the most probable explanation for increased overwintering mortality in infected ladybirds.

1. Introduction

In temperate climates, winter is a period of increased risk for the survival of insects, as they have to face hostile environmental conditions, particularly low temperatures and limited food resources [1,2]. Winter mortality may reach up to 90% in some insect species [2], making overwintering a key phase in their life cycles. Unsurprisingly, a plethora of behavioural and physiological adaptations have evolved in insects to improve their low-temperature performance and minimize energy expenditures during overwintering [2–4]. Most temperate insects undergo a complex process of winter diapause when metabolic rates are minimized and low-temperature stress resistance is maximized [5,6].

The vast majority of studies investigating overwintering success in insects have focused on the effects of abiotic factors, mainly temperature experienced prior to and during winter [4,7–9]. Compared to optimal winter temperature, both decreased and increased temperatures can reduce winter survival and post-overwintering insect performance because of chill injuries and energy reserves exhaustion, respectively [6–9]. In addition, insect resistance to temperature stress is a plastic trait, and thus pre-winter acclimatization can strongly modify winter mortality [4,10,11]. Biotic factors may be as important as abiotic

ones, but this topic has been only rarely investigated. Food availability and quality prior to overwintering was previously identified as an important factor determining overwintering success [6,12], and the potential role of predators, parasites and pathogens has been proposed [13–16].

An insect's immune system can be affected during overwintering as its maintenance is energetically costly and a substantial investment into the immune system can compromise insect survival during winter [17]. However, recorded changes in insect immune parameters during winter are species- and parameter-specific, probably because of complex reasons, including evolutionary history of both the insects and their pathogens or parasites, habitat-specific pathogen or parasite load and species-specific effect of low temperature on pathogen or parasite performance [14,17,18]. Insect immune systems can also be strongly affected by an ongoing infection that can both increase and decrease particular parameters because of immune system inducibility, as well as physiological trade-offs or insect organism exhaustion [18–20]. Thus, pathogen and parasite infections probably modify changes in insect immune parameters during the winter period. However, the complex nature of this phenomenon and missing case studies do not allow us to make any specific predictions about how.

In this study, we investigate the effects of the biotrophic fungus *Hesperomyces virescens* (Ascomycota: Laboulbeniales) on the winter survival and immune system functioning in *Harmonia axyridis*, the harlequin ladybird (Coleoptera: Coccinellidae). *Harmonia axyridis* is native to Asia but was introduced in many countries as a biological control agent and unintentionally spread to many others countries in North and South America, Europe and Africa [21]. Its invasive success is attributed to the combination of high reproductive potential, superior intraguild competitiveness and an efficient immune system [22]. Despite this efficient immune system, several pathogens, parasites and parasitoids are known to exploit *Ha. axyridis*, including *He. virescens*, *Coccipolipus hippodamiae* mites, *Parasitylenchus bifurcatus* nematodes and male-killing bacteria in the genera *Spiroplasma*, *Rickettsia* and *Wolbachia* [23]. Interestingly, *Ha. axyridis* coexists well with selected pathogens (e.g. Microsporidia [22]), and some natural enemies adapted to *Ha. axyridis* as a novel host recently during its ongoing invasion (e.g. the parasitoids *Dinocampus coccinellae* and *Phalacrotophora* spp. [23,24]). It should be noted that Řeřicha *et al*. [16] have shown that the *Ha. axyridis* immune system and ability to respond to bacterial infection are weakened after winter, which can also indicate increased susceptibility to other pathogens during the winter season.

The fungus *He. virescens* is a microscopic obligate ectoparasite of ladybirds [25–27]. It was recently shown that *He. virescens* is a complex of multiple species, segregated by their host species [26]. *Hesperomyces* spp. complete their entire life cycle on the integument of a living host, where individual yellowish-greenish thalli are formed from ascospores [28]. Thalli can be formed on any part of the body of the insect and penetrate the cuticle by formation of a rhizoidal haustorium. However, female ladybirds are typically infected on their elytra and males ventrally on their abdomen during the growing season, which corresponds well to the transmission of *He. virescens* during ladybird mating [29]. During the winter period, both males and females are frequently infected on other body parts, indicating transmission of ascospores in *Ha. axyridis* overwintering aggregations [27,30]. *Hesperomyces virescens* can increase the mortality of infected *Ha. axyridis* individuals in laboratory conditions, mimicking the growing season [31]. However, the effects of infection on winter survival and immune system functioning are unknown.

We hypothesize that *Ha. axyridis* ladybirds infected by *He. virescens* will suffer higher winter mortality and reduced post-overwintering survival resulting from enhanced energy consumption, as entomopathogenic fungi can both directly exhaust ladybird energy reserves [25] and induce energy-cost responses in insect immune systems [32]. Moreover, we expect that fungal infection will result in greater decreases in measured ladybird immune parameters compared to uninfected beetles, resulting from energy exhaustion and parasite-induced immune system suppression in infected ladybirds [33]. In addition, we predict that ladybird winter survival will correlate positively with their pre-overwintering body mass and haemolymph parameters, as both high body mass and investment into immune system are considered to be a proxy for good physiological condition in insects [12,34,35].

2. Material and methods

(a) Experimental set-up

Ladybirds employed as experimental animals in our laboratory experiment were collected in October 2018 as adults aggregating at overwintering sites in Bohemia (Czech Republic). Source populations were originated from České Budějovice (48.9794344 N, 14.4451989 E), Mořina (49.9538697 N, 14.2058467 E), Myštice (49.4540217 N, 13.9681197 E), Nučice (50.0182142 N, 14.2314867 E) and Ohaře (50.0972283 N, 15.3095158 E). The ladybirds were stored in climatic chambers set to a 10 L : 14 D and 12°C : 6°C regime immediately after transportation to the laboratory to avoid interruption of diapause induction. The presence or absence of *He. virescens* was checked for each ladybird using a dissecting microscope at 50× magnification. Ladybirds were sorted into uninfected (no visual signs of fungal infection), slightly infected (1–15 mature thalli) and heavily infected individuals (greater than 15 mature thalli). For the following experiment, only the uninfected and heavily infected individuals were used. Prior to the start of the overwintering experiment, approximate age based on elytral coloration (yellow and pale orange = young, red = old; [29]), sex and pre-overwintering live body mass were determined for all ladybirds. Each ladybird was placed in a separate Petri dish with a watered cotton ball to preclude ladybird desiccation during the winter. Individuals from each source population were evenly distributed among treatments, i.e. infected or uninfected beetles were employed in experiment 1 and experiment 2 (see below). The overwintering experiments started at the end of October (experiment 1) and beginning of November (experiment 2) when the experimental beetles were moved to climatic chambers with conditions mimicking natural winter temperatures. The 'normal' temperature regime used by Knapp & Řeřicha [7] was employed, characterized by fluctuating temperatures with a mean temperature of 0.7°C and a minimum temperature of −4.1°C throughout the winter (November to March; for details, see the electronic supplementary material, table S1).

Two sets of ladybirds were used for two separate experiments. In both experiments, we investigated the effects of fungal infection and individual parameters (autumn body mass, age and sex) on winter survival. In experiment 1, we analysed body mass change (from autumn to spring), and beetles were kept alive after overwintering to investigate their post-overwintering longevity without food. In experiment 2, ladybird haemolymph was

sampled prior to and after overwintering to investigate the effects of fungal infection and individual parameters on autumn values and post-overwintering changes of selected ladybird haemolymph parameters (total haemocyte concentration, total protein concentration, antimicrobial activity against *Escherichia coli* and microsporidia load; see details below). The final datasets consisted of 127 individuals for experiment 1 and 82 individuals for experiment 2; however, it should be noted that final sample sizes for particular haemolymph parameters in experiment 2 can be lower owing to winter mortality or unsuccessful haemolymph sampling after winter (for details, see raw data deposited here: [36]). Note that after overwintering, we visually checked whether uninfected beetles had any signs of *Hesperomyces* infection (i.e. whether thalli had developed during winter), but this was not the case for any of the individuals.

(b) Measurement of response variables

For both experiments, winter survival of experimental beetles was recorded monthly throughout the overwintering period (i.e. at the end of November, December, January, February and March). Live diapausing ladybirds were commonly tightly attached to the substrate (Petri dish), whereas dead ladybirds dropped off and laid freely on their dorsum. Ladybird attachment to Petri dish walls is allowed by special setae on their tarsi [37]. Note that this way of identifying dead individuals is highly reliable, as confirmed by our previous study [7]. Body mass change was computed for experiment 1 as the difference between pre-overwintering and post-overwintering body mass, measured using a Sartorius balance with a precision of 10^{-4} g. Moreover, to control for pre-overwintering body mass effects, proportional body mass change was calculated as the proportion of the autumn body mass that was loss during winter (body mass change/autumn body mass). Prior to both body mass measurements, water was offered to the experimental beetles for 24 h to allow them to optimize the water content of their bodies (in spring, the measurement was performed after 24 h spent at 22°C). In experiment 1, post-overwintering longevity of beetles that survived winter was measured as the time (in days) that they survived at 22°C without any food, but water was provided ad libitum.

To sample haemolymph for measurements of selected haemolymph parameters in experiment 2, beetles were induced to reflex bleed by poking their legs with an entomological pin both prior to and after overwintering (the method is described in [38,39]). The same individuals were sampled before the winter and then resampled after the winter. If the volume of collected haemolymph was lower than 0.5 µl during spring resampling, beetles were also subjected to puncture sampling of haemolymph [38] to maximize the number of samples available for measurement of all the evaluated parameters. For both sampling methods, we collected haemolymph using a glass microcapillary (Hirschmann, Germany), and the sampled amount was measured using a digital calliper with a precision of 0.01 mm. Collected haemolymph was immediately diluted (100× dilution) in anticoagulant buffer (62 mM NaCl, 100 mM glucose, 30 mM trisodium citrate and 26 mM citric acid), and the total haemocyte concentration was recorded immediately using a Bürker chamber under a Carl Zeiss Primo Star microscope (set to 100× magnification). At the same time, the microsporidia load was measured using a Bürker chamber under a Carl Zeiss Primo Star microscope (set to 400× magnification) by counting the number of microsporidia in five small squares per sample. Note that this method just provides relative values because of limited depth of field (it is impossible to focus throughout the sample as alive microsporidia are moving). However, the repeatability of the method is considerably high (intraclass correlation coefficient is close to 0.9; M. Řeřicha 2019, personal observation). Later, the antimicrobial activity of haemolymph against Gram-negative bacteria was measured luminometrically from frozen samples using bioluminescent *E. coli* K12, which contains luxABCDEamp plasmid for expression of bacterial luciferase and its substrate (for methodological details, see [16,40]). Briefly, 40 µl of haemolymph diluted in an anticoagulant buffer (100-fold dilution) were mixed in a reaction well with 120 µl of a bacteria working solution containing 100 000 bacterial cells in a phosphate buffer (pH 7). The luminescence signal, which is positively correlated with viability of *E. coli* K12, was recorded using a Chameleon V luminometer (Hidex, Finland) in counts per second. Finally, total protein concentration in the haemolymph was measured colourimetrically with a Bradford protein assay (Bio-Rad, Hercules, CA). Note that total protein concentration is a general parameter that can be partly related to both antimicrobial activity (as antimicrobial peptides are included among haemolymph proteins; [41]) and energy reserves management [42]. However, a detailed analysis would need the identification and quantification of the peptides included in haemolymph.

(c) Statistical analyses

To analyse winter survival, datasets from both experiments were merged, and generalized linear models (GLMs) with a Poisson distribution were employed, using the number of months that beetles survived during the winter (from 0 to 5) as the response variable. Ladybird sex, age, autumn body mass, fungal infection and their pairwise interactions were included as predictors. Using the same model structure, data from experiment 1 were used to analyse post-overwintering longevity using GLMs (Poisson distribution), body mass change and proportional body mass change (using linear models). Moreover, we performed an additional analysis of winter survival determinants using only data from experiment 2. Similar GLMs were used, but autumn haemolymph parameters were also included as predictors to test for their influence on winter survival.

Using the dataset from experiment 2, effects of ladybird sex, age, autumn body mass, fungal infection and their pairwise interactions on pre-overwintering haemolymph parameters were analysed using similar models (negative binomial GLMs for haemocytes and microsporidia, linear models for protein concentration and antimicrobial activity). To analyse effects of *Hesperomyces* infection and other factors on changes in haemolymph parameters throughout the winter, generalized linear mixed models were used. Pre- and post-overwintering haemolymph parameters were used as response variable, individual identity as a random factor and season (autumn versus spring), ladybird sex, age, autumn body mass, fungal infection and their pairwise interactions as fixed factors. Finally, correlations between haemolymph parameters were tested using Pearson correlation tests, separately for pre- and post-overwintering data.

For all response variables, we started with a full model including all paired interactions between predictors and subsequently removed all non-significant interactions ($p \geq 0.05$), though marginal interactions ($0.05 < p < 0.1$) were kept if they improved model fit (higher R^2). All analyses were performed with R software v. 4.1.0 [43] using the packages *MASS* [44] and *lme4* [45]. Model assumptions were checked with the package *performance* [46], and figures were produced using the package *ggplot2* [47].

3. Results

(a) Winter survival

The results from the combination of both overwintering experiments revealed that fungal infection was the only variable that significantly affected ladybird winter survival (table 1 and figure 1*a*). Winter survival was lower for heavily

Table 1. Effects of sex, age, autumn body mass and fungal infection on winter survival, post-winter longevity, post-winter body mass change and proportional body mass change of *Harmonia axyridis*. (For each response variable, the estimates, *z*- or *t*-values and *p*-values of each predictor are shown (significant *p*-values < 0.05 are highlighted in **bold**). Non-significant interactions that were removed during model simplification are also included (without estimates).)

	winter survival			post-winter longevity			body mass change			proportional body mass change		
predictors	estimates	*z*-value	*p*-value	estimates	*z*-value	*p*-value	estimates	*t*-value	*p*-value	estimates	statistic	*p*-value
intercept	1.62	11.69	<0.001	1.4	4.5	<0.001	−3.48	−2.2	0.03	0.11	2.28	0.025
sex (male)	−0.02	−0.34	0.734	0.06	0.54	0.59	0.33	0.72	0.471	0.01	0.97	0.335
autumn body mass	−0.002	−0.6	0.549	0.01	1.84	0.065	0.3	7.61	**<0.001**	0.002	1.91	0.06
fungal infection	−0.14	−3.26	**0.001**	0.18	1.49	0.136	0.87	1.93	0.057	0.03	1.9	0.061
age (old)	0.04	0.91	0.362	−0.1	−1.1	0.271	0.51	1.09	0.278	0.02	1.16	0.249
sex × fungi	—	−0.222	0.824	−0.39	−2.24	**0.025**	—	−0.663	0.509	—	−0.732	0.466
sex × age	—	−0.350	0.726	—	0.440	0.66	—	−1.346	0.181	—	−1.012	0.314
sex × body mass	—	0.048	0.961	—	1.732	0.083	—	−1.693	0.10	—	−1.406	0.163
age × body mass	—	1.152	0.249	—	1.010	0.312	—	−1.159	0.249	—	−1.303	0.196
age × fungi	—	0.150	0.881	—	−0.149	0.881	—	−1.084	0.281	—	−0.884	0.379
body mass × fungi	—	0.207	0.836	—	−0.334	0.738	—	0.077	0.938	—	−0.527	0.599

infected individuals (60%) than for uninfected individuals (79.8%). Interestingly, none of the haemolymph parameters measured during experiment 2 were good predictors of individual winter survival probability (electronic supplementary material, table S2).

(b) Body mass loss and post-winter longevity

In those individuals from experiment 1 that survived overwintering, body mass loss during overwintering was significantly higher for beetles with higher autumn body mass and was marginally higher for infected individuals compared to uninfected ones (table 1; electronic supplementary material, figure S1). When considering proportional body mass, winter body mass loss also increased with autumn body mass and fungal infection, although the effects of both variables were marginal (table 1 and figure 1).

Post-winter longevity without food was affected by an interaction between fungal infection and sex and, weakly, by autumn body mass (table 1). While longevity of uninfected males and females was similar, heavily infected males lived for shorter periods than females (figure 1*c*). On the other hand, post-winter longevity was higher for beetles with higher autumn body mass, although this link was marginal (electronic supplementary material, figure S2).

(c) Effects of overwintering and *Hesperomyces* infection on ladybird haemolymph parameters

In ladybirds that survived the winter, haemolymph parameters were generally not affected by *He. virescens* infection (table 2). The only exception was antimicrobial activity against *E. coli* that tended to be higher in heavily infected ladybirds compared to uninfected ones (figure 2). By contrast, overwintering significantly affected all measured haemolymph parameters, with the exception of microsporidia load (table 2). Haemocyte and protein concentration as well as antimicrobial activity against *E. coli* decreased from pre-overwintering to post-overwintering samples (figure 2). However, there were no significant interactions between overwintering and infection status (table 2). Microsporidia load was lower in young beetles than in old ones, and it increased in males after overwintering, whereas females had similar numbers in autumn and spring (electronic supplementary material, figure S3). Finally, protein concentration tended to decrease more in females compared to males during overwintering (electronic supplementary material, figure S4).

Separate analyses of pre-overwintering haemolymph parameters (i.e. based on all individuals included in experiment 2) also revealed significant effects of sex, age and an interaction between age and body mass on protein concentration (electronic supplementary material, table S3). Protein concentration was higher in old females than in young females and males, and it decreased with body mass in old individuals but increased in young individuals (electronic supplementary material, figure S5).

Correlations between haemolymph parameters revealed that antimicrobial activity, protein concentration and microsporidia were all positively correlated, both in autumn (figure 3) and after overwintering (electronic supplementary material, figure S6). In autumn, haemocyte concentration was also positively correlated with total protein concentration and microsporidia load, although this correlation was quite weak (figure 3)

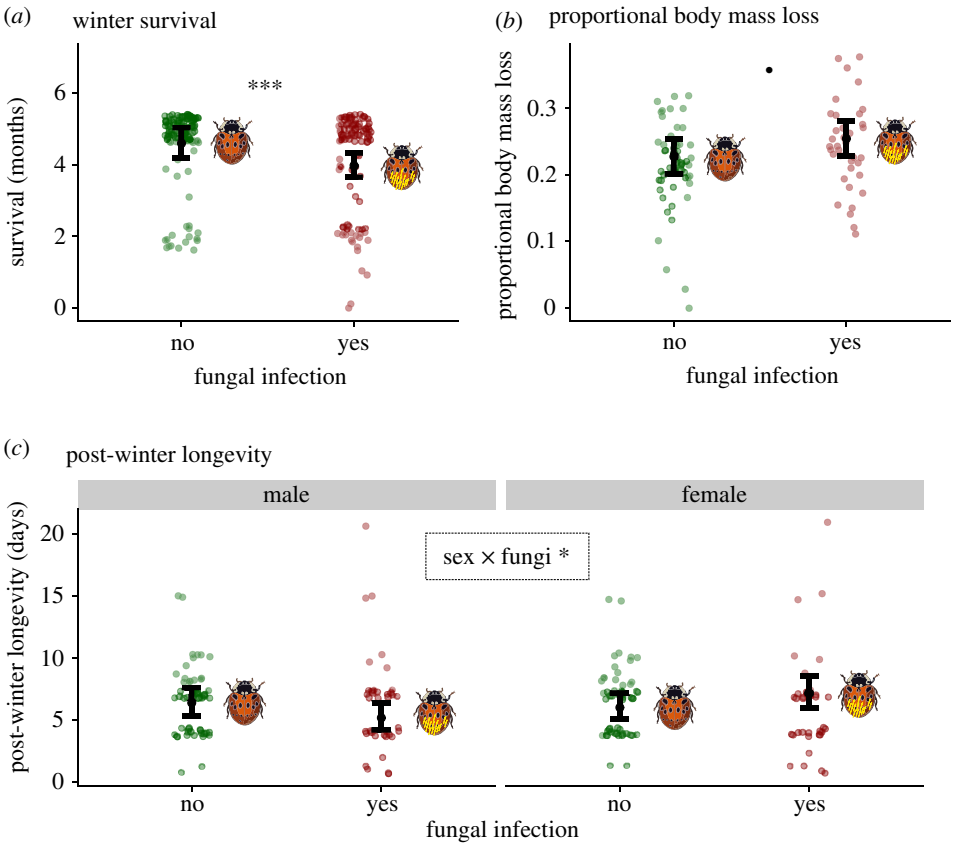

Figure 1. Effects of fungal infection on (*a*) winter survival ($n = 207$) and (*b*) post-winter longevity ($n = 92$), and interactive effects of fungal infection and sex on (*c*) post-winter body mass change ($n = 93$) of *Harmonia axyridis*. For each response variable, the estimates and 95% CI from the models and the raw data for uninfected (green dots) and heavily infected beetles (red dots) are shown. Black dots and stars indicate statistical significance ($\cdot = p < 0.1$; $* = p < 0.05$; $*** = p < 0.001$). (Online version in colour.)

and disappeared completely after overwintering (electronic supplementary material, figure S6).

4. Discussion

Despite the fact that fungal parasites such as *He. virescens* can increase mortality of insects in laboratory conditions mimicking the growing season [31], data on the consequences for overwintering individuals are scarce and limited only to one study by Riddick [48]. Here, we found that fungal infection significantly reduced ladybird winter survival. To our knowledge, this study is the first to investigate the effects of parasitic fungi on changes in haemolymph parameters during insect overwintering. Surprisingly, fungal infection had only weak effects on ladybird immune system. By contrast to haemolymph parameters, ladybird body mass change during overwintering was affected by fungal infection, indicating increased energy consumption in infected ladybirds. Interestingly, pre-overwintering body mass as well as all investigated haemolymph parameters were poor predictors of individual winter survival.

Our study provides additional evidence that *He. virescens* infection has negative effects on ladybird survival, which was proposed by Riddick [48] and shown by Haelewaters *et al*. [31] in their laboratory experiments. In addition, we distinguished between potential mechanisms responsible for increased winter mortality of infected ladybirds. As ladybird age was a poor predictor of ladybird winter survival in our study, we ruled out the possibility that increased winter mortality simply results from ladybird senescence, which can correlate with infection probability in nature [29]. It should be noted that, during the growing season, *He. virescens* infection mainly spreads as a consequence of ladybird mating activity, and during winter, the parasite transmission can be enhanced by the host-aggregating behaviour; thus, infection probability increases with age in promiscuous species such as *Ha. axyridis* [23,27,29,31]. The small effects of *He. virescens* infection on ladybird haemolymph parameters indicate that this parasite causes only a negligible immune challenge to hosts. A possible explanation is that the winter period represents sub-optimal thermal conditions for infection development, and thus, the ladybird immune system is not induced. This explanation is also supported by our unquantified observation that the number of mature thalli on ladybirds decreased rather than increased during the experiment. Unfortunately, knowledge of *Laboulbeniales* thermal biology is very limited [25,49], and further research is urgently needed. An alternative explanation could be that the immune system in some insect species is so strongly affected by physiological processes linked to overwintering that their ability to respond to immune challenges is significantly reduced [34].

Our results suggest that mortality caused by direct costs of immune system, i.e. trade-offs between energy invested into immune system and other processes necessary to body maintenance during winter [34,50], is unlikely. Increased body mass change during winter in infected ladybirds, together with decreased post-overwintering survival without food in males, indicates that energy exhaustion (unrelated to the immune system) is the most probable explanation for

Table 2. Effects of fungal infection, season, sex, age, and autumn body mass on haemolymph parameters of *Harmonia axyridis*. (For each investigated haemolymph parameter, the estimates, *z*- or *t*-values, and *p*-values of each predictor are shown (significant *p*-values < 0.05 are highlighted in **bold**). Non-significant interactions that were removed during model simplification are also included (without estimates).)

	haemocytes			protein concentration			antimicrobial activity			microsporidia		
predictors	estimate	*t*-value	*p*-value	estimate	*t*-value	*p*-value	estimate	*t*-value	*p*-value	estimate	*t*-value	*p*-value
intercept	3.26	10.38	<0.001	53.79	5.77	<0.001	67	7.12	<0.001	3.52	11.43	<0.001
fungal infection	0.11	1.04	0.3	−0.47	−0.14	0.885	6.41	1.94	0.058	0.02	0.14	0.891
sex (male)	0.12	0.93	0.35	−4.19	−0.96	0.341	2.75	0.73	0.467	0.01	0.06	0.956
autumn body mass	0.01	1.13	0.258	0.36	1.35	0.182	0.45	1.68	0.1	0.01	1.23	0.22
age (young)	−0.02	−0.20	0.839	−4.3	−1.22	0.229	−4.21	−1.19	0.239	−0.24	−2.08	**0.038**
season (spring)	−0.63	−7–15	**<0.001**	−28.53	−9.34	**<0.001**	−3.22	−1.84	0.073	−0.04	−0.4	0.691
sex × age	—	2.96	0.09	—	0.43	0.514	—	1.41	0.234	—	2.35	0.125
age × body mass	—	0.47	0.493	—	2.51	0.113	—	0.53	0.467	—	0.02	0.866
sex × fungi	—	0.99	0.318	—	0.08	0.779	—	0.26	0.607	—	0.005	0.945
sex × body mass	—	0.27	0.603	—	1.99	0.158	—	0.16	0.686	—	1.16	0.281
age × fungi	—	0.04	0.848	—	2.72	0.099	—	2.81	0.090	—	3.46	0.063
body mass × fungi	—	0.40	0.528	—	1.58	0.459	—	0.07	0.794	—	0.92	0.339
season × fungi	—	1.49	0.222	—	0.14	0.709	—	1.80	0.179	—	0.59	0.44
season × sex	—	0.98	0.322	—	1.95	0.057	—	0.21	0.644	0.29	2.12	**0.034**
season × age	—	0.34	0.562	—	0.17	0.678	—	0.63	0.426	—	0.43	0.512

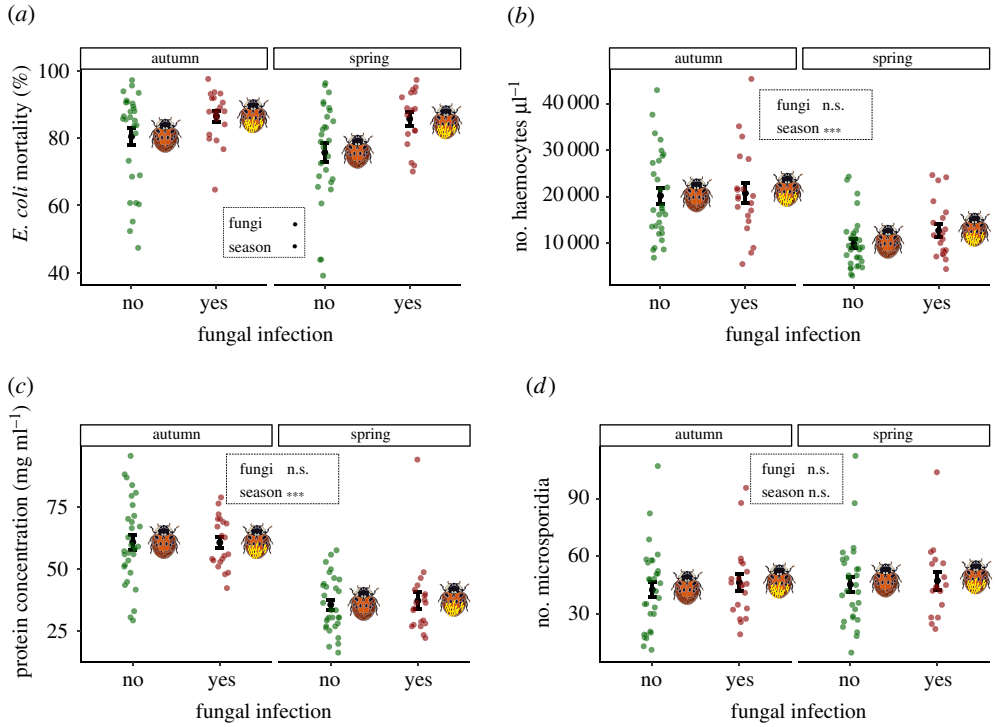

Figure 2. Effects of fungal infection and season on (*a*) antimicrobial activity ($n = 94$, (*b*) haemocytes ($n = 102$), (*c*) proteins ($n = 102$) and (*d*) microsporidia ($n = 102$) in *Harmonia axyridis* haemolymph. For each response variable, mean ± s.e. and raw data are shown for uninfected (green dots) and heavily infected beetles (red dots). Note that the number of microsporidia only represents relative values (for further details, see §2). Black dots and stars indicate statistical significance (n.s. = not significant; · = $p < 0.1$; *** = $p < 0.001$). *n* values represent the complete number of observations, i.e. total number of samples analysed in both autumn and spring. (Online version in colour.)

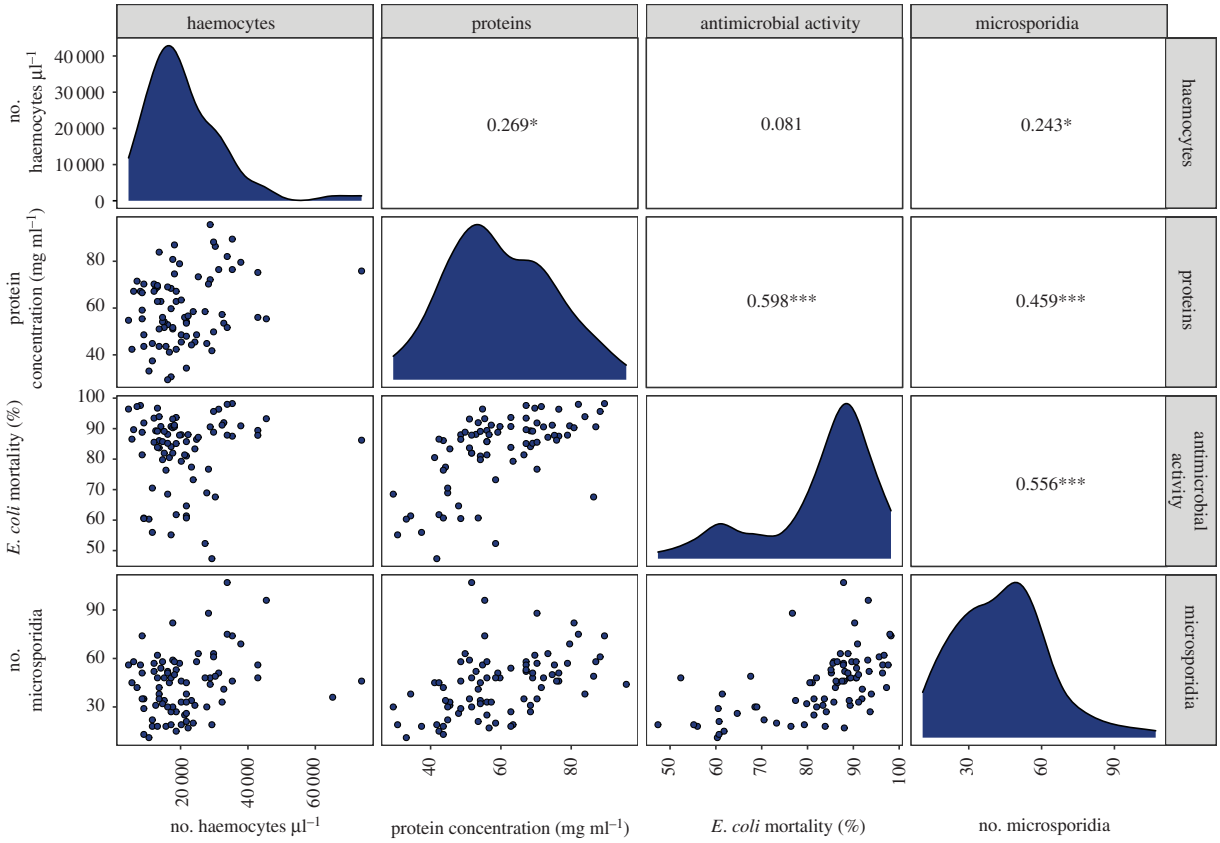

Figure 3. Correlations between pre-overwintering haemolymph parameters. The lower triangle shows scatterplots for each pair of variables, and the upper triangle, the Pearson correlation indices. Stars indicate the significance of correlations (***: $p < 0.001$; *: $p < 0.05$), and numbers represent *r* values. The diagonal panels represent density distributions plots for each variable. (Online version in colour.)

increased overwintering mortality in infected ladybirds. *Hesperomyces virescens* thalli draw nutrients from their ladybird hosts via rhizoidal haustoria penetrating directly into the body cavity and reaching the haemolymph [25,51]. Pre-overwintering energy reserves have been previously identified as a crucial determinant of insect overwintering success [5–7]; thus, it is not surprising that an increased energy reserve consumption rate can increase insect mortality.

Surprisingly, pre-overwintering body mass was not a good predictor of winter survival in our study. One possible explanation is that absolute body mass, *per se*, is not a good proxy for energy reserves available to a given individual; rather, relative body mass (i.e. mass corrected for structural size), or the proportion of substances with high energy content such as lipids, can be a better proxy [52,53]. We also rejected the hypothesis that haemolymph parameters measured prior to overwintering can predict individual winter survival probability in *Ha. axyridis*. General theory predicts that the immune system signals body condition status because individuals in good condition can afford higher investment into immune system development and maintenance [54,55]. However, this pattern can be species-specific [14,34] and less pronounced in species that are exposed to low pathogen and parasite pressure or which are able to employ efficient defensive mechanisms with limited energy costs. The latter can be true for *Ha. axyridis* because its immune system is boosted by presence of the alkaloid harmonine, which has significant antimicrobial activity [56].

On the other hand, at least some costs of *Ha. axyridis* immune system maintenance are manifested by decreased haemocyte and protein concentration values after overwintering and a similar tendency for antimicrobial activity against *E. coli*. Interestingly, the observed decrease was unaffected by infection status; this contrasts with our previous study showing that haemocyte concentration and antimicrobial activity against *E. coli* did not differ between pre-winter and post-winter samples for unchallenged field-collected ladybirds but decreased significantly for *E. coli* challenged beetles [16]. It should be noted that this discrepancy between studies can be partly owing to methodological differences; in the present study, we resampled the same individuals prior to and after overwintering, whereas in our previous study, different sets of individuals were sampled prior to and after overwintering. One possible explanation for the decrease in immune system parameters lies in the reflex bleeding method used for haemolymph sampling during autumn, which can represent a specific type of immune challenge to ladybirds [39]. Alternatively, the fact that ladybirds with lower haemolymph parameters values had a slightly lower winter survival can artificially increase average haemolymph parameters for a group of ladybirds sampled after overwintering, as was performed in Řeřicha *et al*. [16]. Although ladybird survival was not significantly affected by haemolymph parameters in the present study, there was at least a tendency toward this pattern; detailed inspection of our data indicates that individuals with the lowest pre-overwintering antimicrobial activity against *E. coli* did not survive until spring (see also data points in figure 2*c*).

Some of our results are sex-specific, which is not surprising given that sexual size dimorphism, common in many insect species, is also present in *Ha. axyridis* [57,58]. In addition, sexual differences in physiological processes that have evolved owing to sex-specific reproductive roles are also well known [59–61]. For example, males infected by *He. virescens* had reduced post-winter longevity in our study, but females did not. As infected ladybirds tend to lose more mass during overwintering compared to uninfected ones, it is possible that smaller body mass combined with potentially lowered starvation resistance in males (previously reported for another beetle species; [35]) makes infected males more susceptible to spring starvation. More surprising was the finding that changes in microsporidia load during overwintering tend to be sex-specific. Unfortunately, the state of knowledge on microsporidian biology not only limits our understanding of why their numbers tend to increase in males and decrease in females during overwintering but also if they represent parasites or, rather, symbionts responsible for the invasion success of *Ha. axyridis* [22,62]. Finally, ladybird age only affected protein concentration, which is in line with a previous study investigating ontogeny of haemolymph parameters in *Ha. axyridis* [63].

Interestingly, some haemolymph parameters correlated with each other, and these correlations were stronger prior to rather than after overwintering. Total protein content was positively correlated with antimicrobial activity against *E. coli*, which can be explained by the presence of antimicrobial peptides among other haemolymph proteins [41]. Protein concentration and antimicrobial activity were both positively correlated with microsporidia load. It is unclear what allows the existence of high microsporidia loads in *Ha. axyridis* haemolymph, but our findings are in line with previous observations. Steel *et al*. [64] observed positive correlation between microsporidia load and alkaloid content in *Adalia bipunctata* ladybirds. Vilcinskas *et al*. [65] proposed that both antimicrobial peptides and the alkaloid harmonine (which are responsible for bacterial control in *Ha. axyridis*) can reduce tissue damage caused by microsporidia. Future studies are needed to shed light on the interactions between various aspects of *Ha. axyridis* immune system and microsporidia load, propagation, and infectivity.

In conclusion, winter survival of *Ha. axyridis* ladybirds was negatively affected by *He. virescens* infection. Sex-specific effects were observed for ladybird post-overwintering performance as male (but not female) longevity without food was reduced in infected ladybirds. *Hesperomyces virescens* infection only had a minor effect on ladybird haemolymph parameters but tend to cause greater beetle body mass reduction during overwintering, indicating that higher energy consumption is the mechanism responsible for increased ladybird winter mortality. However, further detailed research is needed to fully understand the relationships between *He. virescens* infection and the ladybird immune system. Specifically, exposing uninfected ladybirds and those infected by *He. virescens* to an additional immune challenge after overwintering could help to elucidate if ladybird immune function is really unaffected by this parasitic fungus. Similarly, investigating the effects of *He. virescens* on ladybird immune system during the peak of the growing season, which probably represents optimal growth conditions also for the fungus, can provide valuable novel knowledge on this subject.

Data accessibility. Raw data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.jq2bvq8b6 [36].

Authors' contributions. M.K.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, resources, supervision, writing—original draft, writing—review and editing; M.R.: conceptualization, data curation, investigation, methodology,

writing—review and editing; D.H.: methodology, writing—review and editing; E.G.: data curation, formal analysis, investigation, methodology, validation, visualization, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

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