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Comparative transcriptomics in three ladybird species supports a role for immunity in invasion biology

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ABSTRACT

The spread of the invasive harlequin ladybird (*Harmonia axyridis*) in Europe is accompanied by the decline of the native and non-invasive two-spotted ladybird (*Adalia bipunctata*). Here we show that microsporidia carried by *H. axyridis* can kill *A. bipunctata* following the oral uptake of spores, suggesting that their horizontal transmission via intraguild predation may help the invader to outcompete its native competitor. The native seven-spotted ladybird (*Coccinella septempunctata*) is thought to be less susceptible both to the spread of *H. axyridis* and to its microsporidia. To investigate whether the distinct levels of pathogen susceptibility in these three ladybird species are determined by their immune systems, we compared the immunity-related transcriptomes of untreated beetles and beetles challenged with suspensions of bacteria and yeast. We found that *H. axyridis* carries three and four times as many genes encoding antimicrobial peptides representing the attacin, coleoptericin and defensin families than *C. septempunctata* and *A. bipunctata*, respectively. Gene expression studies following the injection of bacteria and yeasts into beetles revealed that members of these three antimicrobial peptide families are also induced more strongly in *H. axyridis* than *C. septempunctata* or *A. bipunctata*. Our results therefore support the hypothesis that a superior immune system promotes the performance of invasive species.

1. Introduction

Invasive species represent a growing threat for biodiversity and the economy, but it remains unclear why some species are successful invaders whereas others, even closely related ones, are not (Tayeh et al., 2015). The harlequin ladybird (*Harmonia axyridis*) is recognized as a powerful model to evaluate factors that promote invasive success (Roy and Wajnberg, 2008). The native range of *H. axyridis* is central Asia, but it has been introduced into Northern America and Europe as an efficient biological control agent (Lombaert et al., 2011, 2014). It has become an invasive species in these new locations, and its spread is associated with the decline of native competitors such as the two-spotted ladybird (*Adalia bipunctata*) in the United Kingdom (Roy and Brown, 2015). Both *A. bipunctata* and the seven-spotted ladybird (*Coccinella septempunctata*) are predators that compete with *H. axyridis* for food in its newly-colonized habitats (Pell et al., 2008; Kajita et al., 2010). All three species feed on each other's eggs and larvae, a phenomenon known as intraguild predation, which facilitates the horizontal transmission of pathogens among different ladybird species (Saito and Bjørnson, 2006; Vilcinskas, 2015).

Parasites co-introduced with alien species have been postulated to play a role in the outcome of biological invasions (Blackburn and Ewen, 2016). *H. axyridis* carries abundant spores of obligate parasitic microsporidia that do not harm the invasive vector but can kill the native ladybird *C. septempunctata* when injected (Vilcinskas et al., 2013a). To test our hypothesis that the microsporidia carried by *H. axyridis* function like bioweapons against native competitors, and can be transmitted via intraguild predation, we fed *A. bipunctata* larvae with *H. axyridis* eggs, larvae or isolated microsporidia, the latter added to the standard diet comprising eggs of the angoumois grain moth, *Sitotroga cerealella* (Vilcinskas, 2015). The mortality observed in the *A. bipunctata* population following the consumption of *H. axyridis* microsporidia suggests susceptibility to these pathogens in all three ladybird species is determined by their immune systems.

Ecoimmunology has emerged as a novel field which addresses

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also the role of immunity in invasion biology (White and Perkins, 2012). The immune systems of invasive species are thought to be superior to those of closely related non-invasive species (Lee and Klasing, 2004). This hypothesis is supported by the observation that *H. axyridis* is less susceptible to the entomopathogenic fungus *Beauveria bassiana* than the native ladybird species *A. bipunctata* and *C. septempunctata* (Roy et al., 2008). The robust antimicrobial defense of *H. axyridis* relies on both the constitutively present alkaloid (17R,9Z)-1,17-diaminooctadec-9-ene (harmonine) and antimicrobial peptides (AMPs) that are inducible by pathogens (Schmidtberg et al., 2013). Harmonine displays broad-spectrum activity against pathogens including human parasites such as *Leishmania major* and *Plasmodium falciparum* (Röhrich et al., 2012; Nagel et al., 2015). Next-generation-sequencing of the immunity-related transcriptome of *H. axyridis* led to the discovery of more than 50 genes encoding putative AMPs (Vilcinskas et al., 2013b). In order to determine whether this remarkable expansion in the repertoire of immunity-related effector genes evolved within the Coccinelidae, we compared the transcriptomes of untreated *A. bipunctata* or *C. septempunctata* beetles and beetles challenged with microbial pathogens. The resulting datasets were screened for the presence of AMPs and their expression levels before and after microbial challenge were compared to our previous comparative analysis of naïve and challenged *H. axyridis* beetles (Vilcinskas et al., 2013b).

2. Materials and methods

2.1. Insect material and RNA isolation

H. axyridis and *C. septempunctata* adults were collected in and around Giessen, Jena and Ober-Moerlen, Germany, for captive breeding. *A. bipunctata* adults were purchased from BIOCARE GmbH (Urbach, Germany). The rearing of all ladybird species, sample preparation, RNA isolation and the injection of suspensions containing 10 mg/ml lyophilized *Escherichia coli, Micrococcus luteus* and *Saccharomyces cerevisiae* were carried out as previously described (Vilcinskas et al., 2013a,b). RNA integrity was determined using an Agilent 2100 Bioanalyzer and RNA Nanochip (Agilent Technologies, Palo Alto, CA, USA), and RNA quantity was determined using a Nanodrop ND-1000 spectrophotometer.

2.2. Infection with microsporidia

A. bipunctata first to second larval instars were isolated in small Petri dishes and assigned to four groups with different diets: I) untreated *S. cerealella* eggs as a control diet; II) fresh *H. axyridis* eggs; III) *S. cerealella* eggs spiked with microsporidia isolated from *H. axyridis* (17.5×10^8 spores/65 mg eggs); and IV) living *H. axyridis* larvae. For the third diet, hemolymph was isolated from adult *H. axyridis* beetles by cutting the legs at the coxal base and drawing the hemolymph into two volumes of ice-cold anti-coagulant saline. The samples were then centrifuged at 1500 × g for 10 min at 4 °C and the pellets were resuspended in phosphate buffered saline (PBS). The centrifugation and resuspension steps were carried out four times, and 5 μl of the final suspension was added dropwise to 50–100 *S. cerealella* eggs for each *A. bipunctata* larva (Vilcinskas et al., 2013a). During the tests, the different diets were renewed every second day. The survival rates were calculated using Kaplan-Meier survival analysis and the log-rank test in SigmaPlot v11 (Systat Software, Inc., San Jose, CA, USA). Microscopic inspection of dead *A. bipunctata* was performed to confirm the presence of microsporidia.

2.3. Transcriptome sequencing, assembly and annotation

A. bipunctata and C. septempunctata beetles were challenged by injecting a suspension containing 10 mg/ml lyophilized *E. coli, M. luteus* and *S. cerevisiae* as previously described (Vilcinskas et al., 2013a,b). Twenty four hours upon injection of microbial elicitors total RNA was isolated from 10 pooled beetles of each species in the naïve and challenged groups. Transcriptome sequencing was carried out by the Max Planck Genome Center Cologne (MPGCC) on an Illumina HiSeq2500 Genome Analyzer platform using poly(A)$^+$ enriched RNA fragmented to an average of 150 nucleotides. This yielded approximately 30 million paired-end (2 × 100 bp) reads for each of the four samples. Quality control measures, including the filtering of high-quality reads and trimming the read length, as well as *de novo* transcriptome assemblies, were carried out using CLC Genomics Workbench v7.1 (http://www.clcbio.com), by selecting the presumed optimal consensus transcriptome and using previously described parameters (Vogel et al., 2014; Jacobs et al., 2016). The transcriptomes were annotated using BLAST, Gene Ontology and InterProScan searches implemented in BLAST2GO PRO v2.6.1 (www.blast2go.de) as previously described (Vogel et al., 2014). To identify *C. septempunctata* and *A. bipunctata* candidate AMP and lysozyme genes, we established a reference set of known or predicted insect-derived AMPs and lysozymes using published sequences and by searching our in-house database as well as public databases (NCBI). To avoid interpreting incomplete genes or allelic variants as different AMP genes, we used a number of additional filters to obtain a non-redundant set of candidate AMP genes using previously described criteria (Jacobs et al., 2016).

2.4. Sequence submission

We have deposited the short read (Illumina HiSeq2500) data to the European Nucleotide Archive (ENA) with the following study accession numbers: *A. bipunctata*: PRJEB11874 and *C. septempunctata*: PRJEB11877 (EBI short read archive (SRA)). The complete study is also accessible directly at the following URLs: http://www.ebi.ac.uk/ena/data/view/PRJEB11874 and http://www.ebi.ac.uk/ena/data/view/PRJEB11877.

2.5. Analysis of AMP gene expression

Digital gene expression analysis was carried out using CLC Genomics Workbench v7.1 to generate BAM mapping files, QSeq (DNAStar, Inc., Madison, WI, USA) to remap the Illumina reads of each sample onto the corresponding reference transcriptomes, and finally by counting the sequences to estimate the expression levels, using previously described parameters for read mapping and normalization (Vogel et al., 2014; Jacobs et al., 2016). Briefly, the following parameters were used for read mapping: read assignment quality options required at least 50% of the total read bases and at least 90% of bases matching within each read to be assigned to a specific contig; maximum number of hits for a read = 10; mer repeat settings were automatically determined while other settings were not changed.

3. Results

3.1. Infection with microsporidia

A. bipunctata larvae were fed on the four diets described above and observed daily for mortality. The groups fed on *H. axyridis* eggs or first instar larvae died off rapidly (most individuals were dead within 2 weeks) and we determined microsporidia in the cadavers whereas the control group fed on *S. cerealella* eggs survived much

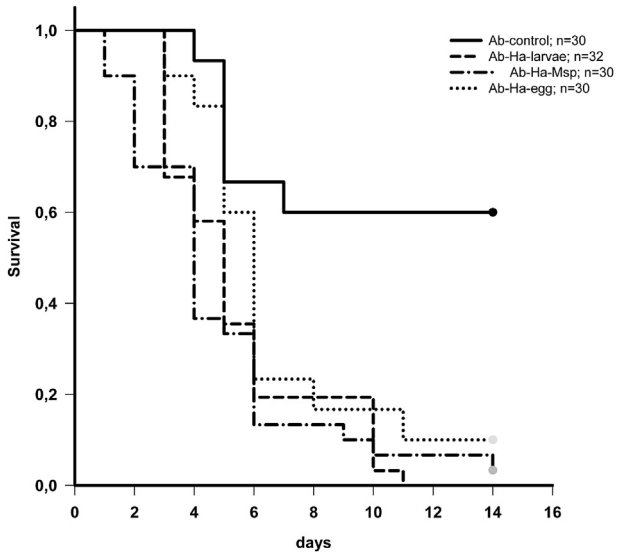

Fig. 1. Survival rate (y-axis) of *A. bipunctata* larval instars (L1/L2) after feeding on *S. cerealella* eggs (Ab-control), *S. cerealella* eggs mixed with microsporidia spores isolated from *H. axyridis* hemolymph (Ab-Ha-Msp), alive *H. axyridis* larvae (Ab-Ha-larvae), and *H. axyridis* eggs (Ab-Ha-eggs). Survival rates were calculated using Kaplan–Meier survival analysis with the log-rank test (Supplementary Material, Table S1).

longer (Fig. 1). Our hypothesis was that the observed accelerated mortality was caused by infection with microsporidia carried by *H. axyridis* (Vilcinskas, 2015). We therefore isolated these microsporidia as previously described (Vilcinskas et al., 2013a) and used them to spike the *S. cerealella* eggs used in the control diet. *A. bipunctata* larvae fed on the spiked eggs died off rapidly whereas those fed on the microsporidia-free eggs survived for much longer (Fig. 1).

3.2. Identification of transcripts encoding AMPs and other immunity-related proteins

RNA-seq analysis was carried out on naïve and challenged *A. bipunctata* and *C. septempunctata* beetles as previously described for *H. axyridis* (Vilcinskas et al., 2013b). We screened the transcriptomes of *A. bipunctata* and *C. septempunctata* for cDNAs encoding AMPs, lysozymes and other immunity-related genes. A comparative analysis of the resulting datasets with the corresponding published data from *H. axyridis* revealed that the attacin, coleoptericin and defensin families had undergone remarkable expansion in *H. axyridis* compared to the native ladybird species (Fig. 2). For example, 16 defensin genes were found in *H. axyridis* but only three in *C. septempunctata* and only one in *A. bipunctata*. The total number of genes representing the three AMP families was 40 in *H. axyridis*, 9 in *C. septempunctata* and 6 in *A. bipunctata*. Five genes encoding putative thaumatin-like genes were found in *H. axyridis* but only two counterparts of these antifungal peptides were identified in the *A. bipunctata* and *C. septempunctata* transcriptomes. In contrast, we observed only minor differences between the ladybird species when we compared the genes encoding cysteine-knot proteins, c-type or i-type lysozymes, and other immunity-related genes including those encoding heat shock proteins or peptidoglycan recognition proteins (Fig. 2).

3.3. Immunity-related gene expression analysis

A comparative analysis of the inducible expression levels of immunity-related genes in the three ladybird species also revealed significant differences between *H. axyridis* and the native ladybird species (Fig. 2). For example, some genes encoding coleoptericins were upregulated by more than 10,000-fold in the challenged *H. axyridis* beetles (Vilcinskas et al., 2013b) whereas the same treatment induces the expression of individual coleoptericins by up to 45-fold in *C. septempunctata* and by only 5-fold in *A. bipunctata*. Interestingly, the injection of microbial suspensions did not induce any defensins in *A. bipunctata*. Furthermore, the challenge induced only minor changes in the expression levels of thaumatins and lysozymes in all three species (Fig. 2).

4. Discussion

We have shown that predation on the eggs or larvae of the invasive ladybird *H. axyridis* is lethal for the native ladybird *A. bipunctata*, but importantly we also provide evidence to confirm our hypothesis that the observed mortality is caused by the

Immune-related Gene Family	*H. axyridis*			*C. septempunctata*			*A. bipunctata*		
	TR-NO	FC	REG	TR-NO	FC	REG	TR-NO	FC	REG
Attacins	10	(100x)	↑	4	(100x)	↑	2	(10x)	↑
Coleoptericins	14	(>1000x)	↑	2	(45x)	↑	3	(5x)	↑
Defensins (-like)	16	(>1000x)	↑	3	(300x)	↑	1	(none)	
Thaumatins	5	(25x)	↓	2	(none)		2	(5x)	↓
Cys-Knot Proteins	4	(10x)	↑	3	(7x)	↑	4	(12x)	↑
Cecropins	2	(none)		1	(none)			n.d.	
C-Lysozymes	4	(2x/6x)	↑↓	3	(none)		4	(2x)	↓
i-Lysozymes	6	(4x)	↑	6	(4x)	↑	7	(8x)	↑

Fig. 2. Total number of *H. axyridis, C. septempunctata* and *A. bipunctata* transcripts encoding AMPs and lysozymes, and their fold changes in expression following an immune challenge. Individual AMP and lysozyme gene families are shown on the left. TR-NO = Number of predicted transcripts for the corresponding AMP family. FC = Fold change in expression in challenged beetles compared to naïve controls. REG = Direction of regulation (red arrows shows induction, green arrows show repression). Data for *H. axyridis* were taken from a previous publication (Vilcinskas et al., 2013b).

abundant microsporidia carried by *H. axyridis* (Vilcinskas et al., 2015). *A. bipunctata* larvae fed on a standard diet of *S. cerealella* eggs were compared to a group reared under the same conditions but fed on eggs spiked with a suspension of isolated microsporidian spores. Most of the latter group died within two weeks while the control group survived. The presence of microsporidia in the cadavers of *A. bipunctata* implicates that horizontal transmission of *H. axyridis* microsporidia by intraguild predation under natural conditions might play a role in the well-documented decline of *A. bipunctata* following the colonization of its habitats by the invasive competitor (Roy and Brown, 2015). Pathogens which are harmless towards the invasive vector but harmful to the native competitor can function like bioweapons, supporting the success of invasive species in newly-colonized habitats (Vilcinskas et al., 2013a). The bioweapon theory predicts that the invasive vector has an immune system superior to that of the native competitor (Vilcinskas, 2015). To test this hypothesis, we compared the immunity-related transcriptomes of three ladybird species which differ in their invasive propensity. *H. axyridis* causes the rapid decline of the indigenous *A. bipunctata* population in newly colonized habitats (Roy and Brown, 2015) whereas a native species (*C. septempunctata*) appears less susceptible to *H. axyridis* and is also invasive in North America.

We focused on the inducible transcription of genes encoding AMPs representing the attacin, coleoptericin and defensin families and observed striking differences between these ladybird species. The *H. axiridis* immunity-related transcriptome contained three times as many genes in these families compared to *C. septempunctata* and four times as many compared to *A. bipunctata*. The number of defensins found in the native species was similar to that reported in the red flour beetle (*Tribolium castaneum*), the only beetle currently with a fully annotated genome, suggesting the defensin gene family has expanded during the evolution of the Coccinellidae. We postulate that the defensins and coleoptericins in *H. axyridis* complement each other to provide broad-spectrum antibacterial defense, because the corresponding *T. castaneum* AMPs were found to act primarily against Gram-positive bacteria when expressed as recombinant proteins (Tonk et al., 2015), whereas *H. axyridis* coleoptericins are active against Gram-negative bacteria (Vilcinskas et al., 2013a, b).

A *T. castaneum* thaumatin displayed strong activity against the entomopathogenic fungus *Beauveria bassiana* when expressed as a recombinant protein (Altincicek et al., 2008). Two thaumatin-like genes were identified in *A. bipunctata* and *C. septempunctata* but we found five in *H. axyridis,* suggesting that the expansion of these antifungal peptide families in the invasive ladybird may contribute to its stronger resistance against *B. bassiana* compared to both native species (Roy et al., 2008). The number of genes encoding c-type and i-type lysozymes was similar in all three coccinellid species. However, recombinant *H. axyridis* c-type lysozymes were found to potentiate the activity of both harmonine and coleoptericin, indicating a synergistic immunity-related function (Beckert et al., 2015), whereas a recombinant *H. axyridis* i-type lysozyme did not show any antimicrobial activity (Beckert et al., 2016).

We compared the expression level of selected AMP genes among the three species before and after challenging the beetles with a microbial suspension. We observed remarkable differences in the induction ratio (the fold change in expression following the challenge) among the species. For example, the coleoptericins, which are specific to the Coleoptera (Mylonakis et al., 2016), were induced by up to 10,000-fold in *H. axyridis*, up to 45-fold in *C. septempunctata* but only by 5-fold in *A. bipunctata*. Both the expansion of immunity-related gene repertoires in *H. axyridis* and the high induction ratio of certain AMP genes in this invasive species compared to native ladybirds provide intriguing examples of the remarkable evolutionary plasticity of insect immunity (Vilcinskas, 2013) and explain higher susceptibility of *A. bipunctata* and *C. septempuncata* against both entomopathogenic fungi (Roy et al., 2008) and the microsporidia carried by *H. axyridis* (Vilcinskas et al., 2013a, b). Here we provided complementary evidence showing a strong increase in the expression of particular families of AMP genes. Taken together, our results suggest that the superior *H. axyridis* immune system helps this species to successfully outcompete native ladybirds in newly-colonized habitats. Our study also supports the hypothesis that the robustness of the immune system determines the performance of successful invasive species.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2016.09.015.

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