Expansion of the antimicrobial peptide repertoire in the invasive ladybird

Harmonia axyridis

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The harlequin ladybird beetle Harmonia axyridis has emerged as a model species in invasion biology because of its strong resistance against pathogens and remarkable capacity to outcompete native ladybirds. The invasive success of the species may reflect its well-adapted immune system, a hypothesis we tested by analysing the transcriptome and characterizing the immune gene repertoire of untreated beetles and those challenged with bacteria and fungi. We found that most H. axyridis immunity-related genes were similar in diversity to their counterparts in the reference beetle Tribolium castaneum, but there was an unprecedented expansion among genes encoding antimicrobial peptides and proteins (AMPs). We identified more than 50 putative AMPs belonging to seven different gene families, and many of the corresponding genes were shown by quantitative real-time RT–PCR to be induced in the immune-stimulated beetles. AMPs with the highest induction ratio in the challenged beetles were shown to demonstrate broad and potent activity against Gram-negative bacteria and entomopathogenic fungi. The invasive success of H. axyridis can therefore be attributed at least in part to the greater efficiency of its immune system, particularly the expansion of AMP gene families and their induction in response to pathogens.

1. Introduction

Invasive species have a negative impact on biodiversity and the economy, but it remains unclear why some species become successful invaders and others, even closely related ones, do not [1–3]. One property that may encourage invasive success is the ability to mount a strong humoral immune response, since this should facilitate the colonization of new habitats containing unfamiliar pathogens [4]. However, immune responses are costly in terms of resources and thus compete with other fitness traits that are essential for invasive success, such as fecundity [5,6].

The role of immunity in invasion biology has been investigated in the harlequin ladybird beetle Harmonia axyridis (also known as the multi-coloured Asian ladybird) because it is an established model invasive species [3]. Harmonia axyridis is native to central Asia but has been introduced into many regions to control aphids and other pests, and has now started displacing native ladybirds and threatening insect biodiversity in general [7]. The invasive success of H. axyridis has been investigated and recent studies have shown that it is better protected against intra-guild predation than native ladybird species [8]. Harmonia axyridis is only sporadically infected by parasitoids and the insects usually survive these attacks [3,7]. In accordance with this hypothesis, H. axyridis is also highly resistant to entomopathogenic nematodes and fungi that tend to kill native ladybirds [9]. For example, native species such as the two-spotted ladybird (Adalia bipunctata) and the seven-spotted ladybird (Coccinella septempunctata) are killed by moderate doses of the fungal pathogen...
Beauveria bassiana, whereas much higher doses are required to kill *H. axyridis* [10]. The components responsible for the robust innate immunity of *H. axyridis* were unknown until we recently discovered a strong constitutive antibacterial activity in the haemolymph of *H. axyridis*, which is absent in *A. bipunctata* and present at much lower levels in *C. septempunctata* [11]. The active compound was identified by mass spectrometry as harmolnine, also known as (17R,9Z)-1,17-diaminoocadec-9-ene, a secondary metabolite with broad-spectrum antibacterial activity in *vitro*, including activity against human pathogens such as Mycobacterium tuberculosis and Plasmodium falciparum [11]. However, harmolnine does not display *in vitro* activity against the fungal pathogen *B. bassiana* at the concentrations found in the haemolymph, suggesting other components of the immune system must confer fungal resistance.

Antimicrobial peptides (AMPs) are known to confer resistance to fungal pathogens [12–14], but little is known about the diversity and activity of these molecules in most insects, making it difficult to determine whether they play a role in invasive success. Forward genetics approaches, such as the analysis of insect transcriptomes, have resulted in the discovery of many immune-related genes and the corresponding networks and pathways involved in pathogen recognition, signal transduction and effector functions, including AMPs [15].

We therefore set out to identify candidate resistance genes in *H. axyridis* by comparing the transcriptomes of beetles injected with bacterial suspensions with those of untreated controls. We identified a number of induced genes encoding AMPs, including two strongly induced AMPs that were active against Gram-negative bacteria and entomopathogenic fungi. We also found a remarkable difference between *H. axyridis* and the model species Tribolium castaneum in the number of paralogues within several AMP gene families. This provides evidence for a two-layered innate immune system in *H. axyridis*, based on AMPs and harmolnine, which may play a role in promoting the invasive success of this species.

2. Material and methods

(a) Insect material and RNA isolation

*Harmonia axyridis* adults were collected in and around Giessen, Jena and Ober-Moerlen, Germany, for captive breeding. The beetles were reared in cages at 26°C and 60 per cent relative humidity with a 16-h photoperiod. Bean plants (*Phaseolus vulgaris*) infested with pea aphids (*Acyrthosiphon pisum*) were maintained as a food source as previously described [16].

We injected 5 μl of bacteria or yeast cells suspended in phosphate-buffered saline (PBS) into the haemocoel of adult beetles 24 h before dissection. The suspensions contained 10 mg ml⁻¹ lyophilized *Escherichia coli*, *Micrococcus luteus* or *Saccharomyces cerevisiae*. Total RNA was obtained from adults and eggs (five individuals per sample) using Trizol reagent (Invitrogen) and was treated with DNase (Turbo DNase, Ambion) to eliminate contaminating genomic DNA. The RNA was purified using the RNeasy MinElute Cleanup Kit (Qiagen) and eluted in 20 μl of RNA Storage Solution (Ambion). RNA integrity was verified 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. Equal amounts of total RNA from egg, beetle and challenged beetle samples were pooled to maximize the number of gene objects and reduce the number of unusable contigs during normalization and sequencing.

(b) Normalization and construction of the cDNA library

Poly(A)⁺ RNA was prepared from the total RNA and used as the template for cDNA synthesis with N6-randomized primers. The cDNAs were then capped with 454 adapters A (5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG-3') and B (5'-CTG AGT CCA AGG CAC ACA GGG GAT GAG-3') and amplified for 18 cycles using a proof-reading enzyme (Phusion DNA polymerase, Thermo Scientific). The optimal number of cycles for double-stranded cDNA synthesis was determined empirically by testing amplification products by electrophoresis to rule out signs of overcycling [17]. Normalization was achieved by one cycle of denaturation and reassociation, resulting in a mixture of single-stranded and double-stranded cDNAs. Reassociated double-stranded cDNA was separated from the normalized cDNA by passing the mixture over a hydroxyapatite column and amplifying the single cDNA strands for 11 cycles [17]. The resulting double-stranded cDNA pool was purified and concentrated using the DNA Clean and Concentrator Kit (Zymogen) and size fractionated on SizeSep 400 spin columns (GE Healthcare) with a cutoff of approximately 150 bp. A proportion of the double-stranded cDNA was cloned in vector pDNR-Lib (Clontech). Bacterial transformation, plasmid mini-preparation, single-pass sequencing of cDNA library clones and sequence assembly were carried out as described [18]. Normalized cDNA material for Roche 454 sequencing was purified for direct sequencing without cloning.

(c) Sequencing, assembly and annotation

The normalized cDNA library was used for 454 pyrosequencing on a Roche 454 FLX instrument and Sanger sequencing on an ABI 3730XL automatic DNA sequencer (PE Applied Biosystems). We sequenced 384 cloned cDNAs using the Sanger method to test the normalization efficiency and cDNA library quality. The pool of cDNAs from the normalization procedure was converted into a Roche 454 sequencing library according to the manufacturer’s protocols, titrated and transferred to microtitre plates for processing at MWG Eurofins. The 454 sequence reads were preprocessed by masking poly(A) tails and removing SMART adapters using custom-written Perl scripts, trimmed for length and quality with standard settings and assembled using the CLC GENOMICS WORKBENCH v. 5.0.1 (CLC bio, Muehltal, Germany) with the following parameters: nucleotide mismatch cost = 2; insertion = deletion costs = 2; length fraction = 0.4; similarity = 0.9; conflict among individual bases resolved by voting for the base with the highest frequency. Contigs shorter than 250 bp were removed from the final analysis. The total number of reads was 1,184,306 with an average read length of 266 bases. The data assembly comprised 48,835 contigs and 27 million bases including 21,454 contigs longer than 500 bases. The largest contig was 6,702 bases in length, and the average contig size was 563 bases.

BLASTx and BLASTn homology searches and functional annotation using gene ontology (GO) terms (http://www.genontology.org), InterPro terms (InterProScan, EBI), Enzyme Commission numbers and metabolic pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) were carried out using the BLAST2GO software suite v. 2.4.1 [19]. A rough estimate of transcriptome coverage was determined by identifying all hits against the conserved KEGG pathway database and an estimate of genome coverage was achieved by identifying the complete ribosomal protein dataset compared with the full set from *T. castaneum*. Based on these findings, the estimated transcriptome coverage for moderately to highly expressed genes...
was near completion (e.g. 78 of 79 T. castaneum ribosomal protein
sequences were identified). Nucleotide sequences were analysed in
more detail using the commercial LASERGENE software package
and BioEdit freeware. Digital gene expression analysis was car-
ried out using the CLC GENOMICS WORKBENCH by
mapping each 454 read to the reference backbone sequence,
which was then used to estimate relative expression levels (aver-
age contig coverage). In order to derive a non-redundant set of
AMPs, we carefully analysed the different H. axyridis AMP
sequences using a more conservative approach and excluded
from the analysis any protein sequences differing by a single
amino acid. We also designed primers specific for each AMP
gene family and gene family member wherever possible, and
used these to amplify H. axyridis AMP cDNAs and to verify
them by standard Sanger sequencing. All of the AMPs used for
our analysis could thus be verified by sequencing individual
clones, eliminating the possibility of chimeric sequences deduced
from potentially false contig assemblies. However, we cannot
rule out the possibility that some of the AMP sequences rep-
resent (rare) allelic variants and are not necessarily different
genomes because environmental heterogeneity and pathogen diver-
sity can maintain host genetic variation in immune function by
favouring the spatio-temporal distribution of alternative geno-
types [20]. On the other hand, this diversity of AMPs could not
be found in any of the other extensive transcriptomic studies
we and others have performed.

(d) Analysis of AMP gene expression
We used quantitative real-time RT–PCR to compare the relative
expression levels of a range of AMP gene belonging to different
AMP gene families in control and immunized adult H. axyridis
beetles and untreated eggs. Total RNA (500 ng) pooled from the
tissues of 10 adults was reverse transcribed with a 3:1 mix of
random and oligo-dT20 primers, and quantitative real-time PCR
was carried out in optical 96-well plates on a Stratagene MX
3000P system using the Verso SYBR Green 2-Step QRT-PCR Kit
Plus ROX Vial (Thermo Scientific) following the manufacturer’s
instructions. The specific amplification of transcripts was verified
by dissociation curve analysis. All primers were designed using
Primer3 (v. 0.4.0). Transcripts encoding eukaryotic initiation
factor 4A and ribosomal protein RP53 were used as references
for normalization. Raw data and cycle threshold values (Ct)
were analysed with QBASE, using fold change of relative
expression level in the immunized beetles compared with the
sham-injected control beetles or control eggs, with lower
expression level in the immunized beetles compared with the
untreated eggs and beetles reared under controlled con-
tions and from beetles challenged by injecting a bacterial
and fungal suspension to induce immune-related genes. Equal amounts of RNA from each sample were pooled and
sequenced using the Roche 454 FLX platform, and 1.1 million
reads were assembled de novo to produce approximately
48 000 contigs. These were used as BLASTx and BLASTn
search queries to identify homologues in other species, and
functional annotation was carried out using the BLAST2GO
software suite to match unique genes against GO terms, InterPro terms, EC numbers and KEGG metabolic pathways.
A digital gene expression analysis was performed by
mapping the individual 454 reads back to the reference back-
bone (contig sequences). The most highly expressed genes (top
200 contig sequences with highest read coverage) in the
moderately normalized H. axyridis library encoded proteins
involved in the mitochondrial respiratory chain and ATP syn-
thesis (cytochrome c, vacuolar ATP synthase, electron transfer
flavoprotein), general cellular homeostasis, ribosomal proteins
and also many immune effector genes (see the electronic sup-
plementary material, file S1). An extensive search for cDNAs
encoding AMPs and lysozymes resulted in the identification of
a remarkably large and diverse spectrum of AMPs, vastly
exceeding the number found in other insect species (figure 1).

(e) Sequence alignment and
phylogenetic reconstruction
Amino acid sequences were aligned using CLUSTALW v. 2 [21] or
MAFFT [22] and visually inspected for regions of high-quality
alignment. Phylogenetic reconstruction was carried out using
Bayesian inference (MrBayes v. 3.1) or PhyML on the web-based
phylogeny server (http://www.phylogeny.fr). For Bayesian
analysis, the prior was set for the amino acid models to mix,
thereby allowing model jumping between fixed-rate amino acid
models. Markov chain Monte Carlo runs were carried out for
10 000 000 generations after which log-likelihood values showed
that equilibrium had been reached after the first 5000 generations
in all cases, and those data were discarded from each run and
considered as ‘burnin’. Two runs were conducted for the dataset
showing agreement in topology and likelihood scores. The
maximum-likelihood and Bayesian tree topologies were in agree-
ment, including their general subfamily relationships and node
supports. The gene trees were visualized and optimized with the
MEGA5 v. software package [23]. All of the deduced AMP protein
sequences from H. axyridis, other Coleopteran and insect ESTs and
NCBI sequences used in the phylogenetic analysis can be found in
the electronic supplementary material, file S1.

(f) Antimicrobial assays
Haxy_Col1 and Haxy_Col6 were dissolved in equal volumes of
dimethylformamide (Sigma) to a final concentration of
50 mg ml⁻¹ (Haxy_Col6) or 80 mg ml⁻¹ (Haxy_Col1). Gram-
positive M. luteus and Gram-negative E. coli D31 were
maintained on LB agar plates (Carl Roth). Bacteria in their expo-
growth phase were diluted in LB broth to OD600 = 0.02 and
supplemented with the AMPs. At least three measurements of
bacterial growth were performed. The optical density at 37°C
was measured at 20 min intervals. After 24 h, the samples were
serially diluted in PBS and plated on LB agar. The colony-
forming units were counted after an overnight incubation at
37°C. The fungus Metarhizium anisopliae strain 43 (obtained
from the Julius-Kühn-Institute, Darmstadt, Germany) was main-
tained on potato dextrose agar (Carl Roth) and conidia were
isolated as described [24]. Approximately 16 000 conidia
were inoculated in potato dextrose broth (Carl Roth) supplemented
with the AMPs and incubated at 27°C. The germinating conidia
were counted 22 h later under an optical microscope (Leica).

3. Results and discussion

(a) Identification of Harmonia axyridis transcripts
encoding antimicrobial peptides and other
immune-related proteins
We initiated a next-generation transcriptome sequencing pro-
gram for H. axyridis to make up for the lack of publically
available sequence data. Total RNA was isolated from
untreated eggs and beetles reared under controlled con-
tions and from beetles challenged by injecting a bacterial
and fungal suspension to induce immune-related genes. Equal amounts of RNA from each sample were pooled and
sequenced using the Roche 454 FLX platform, and 1.1 million
reads were assembled de novo to produce approximately
48 000 contigs. These were used as BLASTx and BLASTn
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involved in the mitochondrial respiratory chain and ATP syn-
thesis (cytochrome c, vacuolar ATP synthase, electron transfer
flavoprotein), general cellular homeostasis, ribosomal proteins
and also many immune effector genes (see the electronic sup-
plementary material, file S2). An extensive search for cDNAs
encoding AMPs and lysozymes resulted in the identification of
a remarkably large and diverse spectrum of AMPs, vastly
exceeding the number found in other insect species (figure 1).

(i) Attacins
Ten genes encoding attacins were found in the H. axyridis
transcriptome (figure 1), compared with three identified in the T. castaneum genome. Attacins were first discovered
in the silk moth *Hyalophora cecropia* (family Attacidae) and are characterized by a molecular mass of approximately 20 kDa and a high content of glycine residues [25]. Attacins bind to bacterial lipopolysaccharides; therefore, basic attacin is more active against *E. coli* than the acidic form. Further, attacin reportedly blocks the translation of genes encoding outer membrane proteins when it binds to the bacterial cell surface [25]. Interestingly, allelic divergence and paralogous gene conversion has been reported among the attacin genes of *Drosophila melanogaster* [26]. Whether or not these mechanisms caused the diversification of attacins in *H. axyridis* will be addressed in future work.

(ii) Coleoptericins

Fifteen genes encoding coleoptericins and four encoding coleoptericin-like AMPs were identified in the *H. axyridis* transcriptome (figure 2), which is a significant expansion compared with the two coleoptericins encoded in the *T. castaneum* genome [27]. Coleoptericins are structurally similar to attacins and are rich in glycine and proline residues. The first coleoptericin was discovered in *Allomyrina dichotoma* larvae challenged with bacteria [28], and a further member of this protein family has been identified in the burying beetle *Nicrophorus vespilloides* [29]. The deduced amino acid sequences of all the *H. axyridis* coleoptericins include a signal peptide for extracellular localization and the conserved furin cleavage site, yielding a mature peptide approximately 75 amino acids in length (see the electronic supplementary material, figure S1). Although the four *H. axyridis* coleoptericin-like proteins also contain these conserved motifs, the predicted mature peptides are approximately 15 amino acids shorter than the coleoptericins identified thus far, resulting in a clear phylogenetic separation (figure 2). The activity of coleoptericin peptides against Gram-negative bacteria (including methicillin-resistant *Staphylococcus aureus*) is not mediated by the formation of membrane pores but by interference with cell division [28]. Interestingly, a recent study provides evidence that the weevil *Sitophilus zeamais* uses coleoptericins to maintain control over their endosymbiotic bacteria, although it is unclear whether *H. axyridis* uses a similar strategy [30].

(iii) Defensins

At least 19 genes encoding defensins were identified in the *H. axyridis* transcriptome (figure 1) compared with the four defensin and defensin-like AMPs identified in the *T. castaneum* genome using bioinformatics and experimental approaches [27,31]. Defensins are cationic AMPs found in plants, fungi and animals, which are characterized by a triple-stranded β-hairpin structure and six conserved disulphide-linked cysteine residues [32]. The phylogenetic analysis of insect defensins suggests there are three subfamilies in insects [33]. The *H. axyridis* defensin genes we identified are induced following a bacterial challenge (see the electronic supplementary material, figure S2), in agreement with similar experiments in other beetles [29,31]. Most insect defensins display antibacterial activity whereas some, such as gallerimycin from the greater wax moth *Galleria mellonella*, act exclusively against mycelium-forming fungi [12].

(iv) Thaumatins

We identified four genes encoding thaumatins in the *H. axyridis* transcriptome, the same number identified in *T. castaneum* [31]. Thaumatins are frequently found in plants, and have also been identified in the nematode *Caenorhabditis elegans* [34] and in the pea aphid *A. pisum* [35]. These AMPs are larger and more complex than attacins and coleoptericins, with approximately 200 amino acid residues and multiple disulphide bridges. The potent antifungal activity of *T. castaneum* thaumatin was preserved when a recombinant version was tested *in vitro* [31].

(v) Lysozymes

We identified four chicken-type (C-type) and six invertebrate-type (I-type) lysozyme genes in the *H. axyridis* transcriptome, which is similar to or higher than the number found in other
depression was no worse than that suffered by native

years have largely arisen by gene duplication after speciation. The

populations showed that although

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N. vespilloides clustered

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insects, depending on the species [36]. C-type lysozymes
degrade peptidoglycans in the bacterial cell wall by hydro-

ing the β(1–4) linkages between N-acetylglucosamine and N-acetylmuramic acid residues, hence their predominant activity against Gram-positive bacteria [32]. However, the C-type lysozyme from the greater wax moth G. mellonella also shows moderate activity against Gram-negative bacteria and fungi [37]. I-type lysozymes contain five or six disulphide bonds, explaining their thermostability and resistance against proteolytic degradation, but their biological function remains to be determined [36]. Phylogenetic analysis clustered the four C-type lysozymes from H. axyridis and the five from T. castaneum, but indicated that gene duplication events had occurred after speciation. Likewise, the six I-type lysozymes from H. axyridis and the four from N. vespilloides clustered together but gathered in species-dependent subclusters (see the electronic supplementary material, figure S3), which would indicate that the majority of I-type lysozymes evolved by gene duplication events preceding speciation.

(b) AMP diversity in Harmonia axyridis and comparison to other immunity-related genes

It has been proposed that, depending on the population size and diversity, invasive species in new environments should go through a population bottleneck, reducing the levels of heterozygosity and potentially leading to inbreeding depression. However, a recent comparison of native and introduced H. axyridis populations showed that although the introduced populations experienced a population bottleneck of intermediate intensity, the resulting inbreeding depression was no worse than that suffered by native populations, perhaps because deleterious alleles were purged thus promoting high fitness even when inbred [38]. This would suggest that the limited genetic variation in introduced H. axyridis populations would increase the mean fitness relative to more genetically diverse native populations. This limited genetic variation should on the other hand also lead to reduced maximum numbers of alleles (including those derived from genes coding for AMPs) identified in the invasive H. axyridis population used for our studies. However, although this is true for almost all of the transcripts we analysed (including immune-related ones), the AMP-related transcripts violate this hypothesis.

The genome of the reference beetle T. castaneum appears to encode at least 16 AMPs and four lysozymes when experimental and bioinformatics data are combined [27,31]. In contrast, the immunity-related transcrptome of H. axyridis contains a slightly larger number of lysozyme genes but a vastly expanded repertoire of at least 50 AMP genes representing several different AMP families. It would be preferable to compare the immune gene repertoire of H. axyridis directly with those from non-invasive ladybirds such as C. septempunctata but there is insufficient published sequence data for such comparisons, making T. castaneum the best reference genome currently available. Although this may present drawbacks in terms of the taxonomic distance between the species and their different diets and lifestyles, the fact that most immunity-related genes are present in similar numbers in both species and only certain AMP families have expanded in H. axyridis suggests that the comparison is nevertheless valid. The diversity in both cases appears to have largely arisen by gene duplication after speciation. The remarkably dynamic evolution of AMPs in insects is likely

Figure 2. Gene phylogeny, gene expression and activity of H. axyridis coleoptericin proteins. (a) Maximum-likelihood phylogenetic tree of H. axyridis coleoptericin (Haxy_Col) and coleoptericin-like (Haxy_ColL) proteins. (b) Differential gene expression in H. axyridis immune-induced beetles compared with control beetles is depicted for a selected set of coleoptericins and coleoptericin-like genes. Values are shown as immune-induced versus control beetle fold changes with s.d. shown in brackets. Higher fold-change values are depicted in darker grey, whereas lower changes in gene expression are depicted in light colours. (c) Functional assay of two coleoptericin peptides displaying activity against entomopathogenic fungi.

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were repeated two times with similar results. 

Figure 3. Activity of *H. axyridis* coleoptericins against bacteria. Growth of *E. coli* (*a*,*b*) and *M. luteus* (*c*,*d*) in the presence of coleoptericins (BR021 and BR023) from *H. axyridis*. Bacteria were cultured in LB broth supplemented with BR021 (50 µg ml⁻¹) and BR023 (80 µg ml⁻¹), and the optical density was measured every 20 min at 600 nm (*a*,*c*). The bacterial cultures after 24 h were serially diluted and grown on LB agar for the estimation of colony-forming units (*b*,*d*). Experiments were repeated three times with similar results.

to represent successful adaptations to a changing pathosphere [15], although there is no consensus on how such gene duplications are fixed and maintained in the genome [39].

We compared the expansion found in several of the AMP gene families to other immunity-related genes, such as pattern recognition receptor genes (GNBPs and PGRPs). This revealed that the number of paralogues was similar in *H. axyridis* to the reference beetle *T. castaneum* (five GNBPs and four PGRPs), suggesting that some AMP-encoding gene families are potentially more versatile than proteins that bind to microbial cell wall components. Our observation supports the hypothesis that genes encoding immunity-related effector molecules are more evolutionarily dynamic than genes encoding signalling or pathogen recognition proteins, reflecting their ability to adapt more rapidly [15].

(c) Immune gene expression analysis

As well as showing the most remarkable phyllogenetic expansion, the AMP genes were well-represented among the top 200 most strongly expressed genes in the *H. axyridis* transcriptome, calculated by mapping individual reads against the contigs generated by de novo assembly (see the electronic

Figure 4. Activity of *H. axyridis* coleoptericins against entomopathogenic fungi. Germination rate of *M. anisopliae* conidia in the presence of coleoptericins (BR021 and BR023) from *H. axyridis*. The fungus was cultured in PDB supplemented with BR021 (50 µg ml⁻¹) and BR023 (80 µg ml⁻¹), and the germination rate was measured 22 h after inoculation. Experiments were repeated two times with similar results.
investigate their activities against a spectrum of pathogens. The strong induction of a subset of the H. axyridis (d) Coleoptericin activity assays untreated immune induction [36]. At least one of the C-type lysozymes usually displays a strong induction above, while the rest were either unaffected or downregulated. The constitutive synthesis of these AMPs confers by the presence of AMPs. Therefore, we postulate that the harmonine-based constitutive defence system is in line with a recent study using the beetle Harmonia axyridis eggs are known to possess a strong antibacterial activity [11] but our data suggest this is not conferred by the presence of AMPs. We synthesized the predicted mature peptides encoded by the two most strongly expressed AMPs, which were both coleoptericins (Haxy_Col1 and Haxy_Col6). The mature peptides differed at eight amino acids (see the supplementary material, file S2). Indeed, the strongly expressed genes were dominated by three AMP families: the attacins, coleoptericins and defensins. When relative expression levels were presented as average coverage values (which combines the length of the contig with the number of mapped sequence reads), we found that five attacins, six coleoptericins and six defensins were among the transcripts with 150 or more read coverage, similar to strongly expressed housekeeping genes such as those encoding NADH dehydrogenases, cytochrome oxidases and ribosomal proteins.

A subset of the AMP and lysozyme gene was analysed in more detail by quantitative real-time RT–PCR to determine the induction ratio in challenged insects. Several of the coleoptericin-like and defensin family AMPs (and one lysozyme gene) showed moderate induction (2–10-fold), but certain coleoptericins (e.g. Haxy_Col6 and Haxy_Col11), attacins and defensins were induced several hundred to thousand-fold following bacterial challenge (figure 1), consistent with their ranking in the top 200 most strongly expressed H. axyridis genes. It is noteworthy that induction was not strictly AMP family-dependent, e.g. whereas some coleoptericins were strongly or moderately induced, others such as Haxy_ColLD were not induced at all under our experimental conditions. None of the thamaatins were induced in the challenged beetles and some were even downregulated, which is also consistent with the absence of any thamaatin genes in the top 200 ranking. Similarly, only one of the C-type lysozymes genes was moderately induced as noted above, while the rest were either unaffected or downregulated. However, this contrasts with data from other insects where at least one of the C-type lysozymes usually displays a strong immune induction [36].

We also tested the expression of AMPs and lysozymes in untreated H. axyridis eggs. All of the AMPs and all but one of the C-type lysozymes were expressed in the eggs at levels close to or below the detection threshold, whereas the control housekeeping transcripts (EIF-4a and RPS3) were expressed at similar levels in eggs and adults with and without induction. Harmonia axyridis eggs are known to possess a strong antibacterial activity [11] but our data suggest this is not conferred by the presence of AMPs.

(d) Coleoptericin activity assays

The strong induction of a subset of the H. axyridis AMP gene repertoire in response to bacterial challenge led us to investigate their activities against a spectrum of pathogens.

We synthesized the predicted mature peptides encoded by the two most strongly expressed AMPs, which were both coleoptericins (Haxy_Col1 and Haxy_Col6). The mature peptides differed at eight amino acids (see the supplementary material, file S3 and figure S4). The synthetic peptides were tested against a Gram-negative bacterium (E. coli), a Gram-positive bacterium (M. luteus) and an entomopathogenic fungus (M. anisopliae). Haxy_Col1 and Haxy_Col6 inhibited the growth of Gram-negative bacteria strongly, with Haxy_Col6 showing the stronger activity of the two AMPs (figure 3a,b). However, they showed only moderate (Haxy_Col6) to low (Haxy_Col1) activity against Gram-positive bacteria (figure 3c,d). Remarkably, the same AMPs achieved strong (Haxy_Col1) to complete (Haxy_Col6) inhibition of the germination of the entomopathogenic fungus M. anisopliae (figure 4).

A coleoptericin has recently been shown to play a central role in host–symbiont interactions in the weevil S. scirrhosa [30]. The vertically transmitted endosymbionts are prevented from spreading and invading non-bacteriome tissues through the action of a specific AMP, coleoptericin-A, which selectively targets endosymbionts within the bacteriome and regulates their growth by inhibiting cell division. RNAi-based silencing of the coleoptericin gene prevented growth regulation and allowed the endosymbionts to escape and spread into insect tissues [30]. This study, among others, shows that AMPs can have effects other than the direct killing of bacterial and fungal pathogens. Few studies have tested individual AMPs against a broad spectrum of microbial species and strains. However, the growing cumulative evidence from reports on the sometimes highly variable activities of AMPs would support the idea that a highly diverse set of such antimicrobial effector proteins should allow the innate immune system to overcome a broad spectrum of emerging pathogens [15] that invasive species encounter in newly colonized habitats. The constitutive synthesis of these AMPs would require the allocation of resources from other traits relevant for invasive success, such as fecundity, the ability to cope with intra-guild predators or to adapt to different diets. Therefore, we postulate that the harmonine-based constitutive antimicrobial activity [11] is associated with a smaller fitness penalty and adds to a two-layered defence system that combines constitutive chemical defence with inducible AMP-based immune responses (figure 5). The proposed two-layered defence system is in line with a recent study using the beetle Tenebrio molitor, which implies that induced AMPs help to protect the insect host against bacteria that persist despite...
constitutive immune responses, rather than to clear microbial infections [40].

4. Conclusions

The invasive ladybird *Harmonia axyridis* has a two-layered innate immune system, combining a constitutive chemical defence strategy based on the secondary metabolite harmine (which is effective against a range of bacteria) and a broad range of inducible AMPs and defence enzymes (which are effective against both bacteria and entomopathogenic fungi; figure 5). The invasive success of *H. axyridis* may reflect both the robustness and flexibility of its antimicrobial defence that combines constitutive with inducible components. Particularly, the unprecedented diversity of its AMP repertoire which appears to result from multiple gene duplication and divergence events after speciation may contribute to the ability of *H. axyridis* to successfully fight pathogens both in its native range and in newly colonized habitats.

A.V. and H.V. conceived and designed the study, analysed the data, performed sequence alignments and phylogenetic analyses and wrote the manuscript. K.M. performed the antimicrobial assays and parts of the data analysis and helped in writing the manuscript. All of the authors have read and approved the final version of the manuscript.

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References


