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ORIGINAL RESEARCH

A Well Protected Intruder: The Effective Antimicrobial Defense of the Invasive Ladybird *Harmonia axyridis*

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Abstract The harlequin ladybird *Harmonia axyridis* (Coleoptera: Coccinellidae) is a polyphagous predatory beetle native to Central and Eastern Asia. Since 2007 it has established all over Central Europe. In order to elucidate which defense strategy is responsible for its high resistance to diseases, we tested hemolymph as well as eleven main components of the headspace of *H. axyridis* for antimicrobial activity against Gram-positive (*Bacillus subtilis, B. thuringiensis* ssp. *tenebrionis, Micrococcus luteus*) and Gram-negative bacteria (*Escherichia coli*) and yeast (*Saccharomyces cerevisiae*). While three of the volatile compounds weakly reduced the growth of microorganisms, hemolymph of adults and larvae of *H. axyridis* strongly inhibited the growth of Gram-positive and Gram-negative bacteria as well as yeast. Furthermore, we compared the antimicrobial activity in the hemolymph of *H. axyridis* and *Coccinella septempunctata*. Antimicrobial activity in *H. axyridis* was about a thousand times higher compared to hemolymph from *C. septempunctata*. In contrast to *C. septempunctata*, the antimicrobial activity in *H. axyridis* was present without prior challenge. Minimal inhibitory concentration (MIC) of the hemolymph of *H. axyridis* was lowest against *E. coli* and yeast followed by *B. subtilis*, and was highest against entomopathogenic *B. thuringiensis* ssp. *tenebrionidae*. Furthermore, MIC values of the hemolymph obtained from live beetles were significantly lower than from frozen insects. This suggests that the active antimicrobial compound is affected by freezing and subsequent thawing of the beetles. Potential implications of our findings for the competitive advantages of *H. axyridis* over *C. septempunctata* are discussed.

Key Words *Harmonia axyridis* · *Coccinella septempunctata* · Antifungal Activity · Minimal Inhibitory Concentration · Headspace · Lysozyme · Benzaldehyde · Phenol · (+)-Alpha-Pinene · Coleoptera

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Introduction

The introduction of exotic species as biological control agents can have negative effects on the community composition and interactions of native and established species. This could lead to species replacement and extinction (Soares et al., 2008). If the exotic species easily adapts to new habitats, it can become a successful invader. Such an example is *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), the harlequin or multicolored Asian ladybird (Roy and Wajnberg, 2008). This species is a polyphagous predatory coccinellid native to central and eastern Asia. It has been used widely as a biological control agent of aphids in America and Europe (Koch, 2003; Pervez and Omkar, 2006). Besides predation on aphids and other insects, this ladybird species also feeds on a variety of fruits in late summer and fall (Koch, 2003;

Gross et al., 2010). Since its establishment in the 1980s, it has become the dominant ladybird in much of the USA and Canada (Koch and Galvan, 2008). One decade later, it was introduced into Europe and has now colonized 13 European countries (Brown et al., 2008).

One important characteristic of the harlequin ladybird is its high resistance to pathogens and predators. It was shown recently that adult *H. axyridis* were less susceptible to the entomopathogenic fungus, *Beauveria bassiana*, than the native European species, *Adalia bipunctata* and *Coccinella septempunctata* (Roy et al., 2008). *Harmonia axyridis* also was not a suitable host for the reproduction of various entomopathogenic nematodes (Shapiro-Ilan and Cottrell, 2005). Further, lower levels of parasitism by the wasp *Dinocampus coccinellae* were reported for *H. axyridis* compared to *C. septempunctata* (Koyama and Majerus, 2008).

In the immune system of insects, antimicrobial peptides are synthesized *de novo* following an infection (Boman and Hultmark, 1987). In recent years, due to the growing resistance of microbes to antibiotics, a number of studies have focused on the presence and effectiveness of antimicrobial peptides in insect hemolymph. Several of these peptides have been isolated and sequenced (Bulet and Stöcklin, 2005). Most studies involved model insects like *Drosophila melanogaster* and *Galleria mellonella* (Schmid-Hempel, 2005; Brown et al., 2009), but little is known about the disease resistance of Coleoptera, especially coccinellid beetles (Bulet et al., 1991; Gross et al., 2008).

In order to understand the rapid spread of *H. axyridis*, we evaluated several potential mechanisms that underlie the observed disease resistance of this species. As the survival of juvenile stages is one key factor for the successful establishment of invasive populations (Marco et al., 2002), we compared the antimicrobial efficacy of larvae and adults in *C. septempunctata* and *H. axyridis*. Antimicrobial defense of leaf beetles can be exerted either by the innate immune system or by volatile fumigants (Gross et al., 2008; Gross and Schmidtberg, 2009). Thus, we tested the hemolymph as well as several volatile components emitted by *H. axyridis* for antimicrobial potential. Furthermore, we investigated the mode of action of antimicrobial activities by determination of the minimal inhibitory activities (MIC) and activity measurement of key-enzymes of hemolymph obtained from live and frozen beetles.

Methods and Materials

Insects For establishing a laboratory rearing culture of *Harmonia axyridis* (Coleoptera: Coccinellidae), adults were collected in October 2008 in the surroundings of Dossenheim, Germany and reared permanently under controlled conditions in a climate chamber (24°C day, 19°C night, 60% humidity, 16:8 hr/L:D) at the Julius Kühn Institute, Dossenheim. Insects were kept in rearing cages (40×30×30 cm) and fed a diet of pea aphids (*Acyrthosiphon pisum*) reared on beans (*Vicia faba*), pollen (Ullmann, Erlensee, Germany), and water *ad libitum*. Eggs from *Coccinella septempunctata* (Coleoptera: Coccinellidae) were purchased (Katz Biotech, Baruth, Germany), and the beetles reared permanently under the same conditions. For all experiments, we used laboratory reared beetles (larvae and adults).

Microorganisms Bacillus subtilis, B. thuringiensis subsp. *tenebrionis* (strain 10 BI 256–82, isolated from *Tenebrio molitor* (Coleoptera: Tenebrionidae), Krieg et al., 1984), and *Micrococcus luteus*, were chosen as Gram-positive bacteria, and *Escherichia coli* (K12/D31) was used as Gram-negative organism. The yeast *Saccharomyces cerevisiae* (DSM 70499) was tested as an example for fungi (Gross et al., 1998, 2002). All organisms were obtained from the strain collection at the Julius Kühn Institute, Dossenheim. Bacteria were cultivated on Standard 1 agar plates (Roth, Germany) at 28°C (*Bacillus* and *Micrococcus*) or 37°C (*E. coli*), and the yeast on Sabouraud agar (Roth, Germany) at 28°C.

Agar Diffusion Assay

Insect Treatments Fourth instars and adults of both ladybird species were injected with 0.5 µl of sterile water or a dense (~10^{10} cells/ml) heat-inactivated (65°C, 60 min) suspension of *E. coli* with a microapplicator (Burkard, Germany) laterally into the abdomen 24 hr prior to hemolymph collection. A third group of larvae and adults served as untreated control.

Hemolymph Collection Hemolymph was collected from fourth instars or adults. Twenty-four hours after treatment, insects were pressed gently between two glass slides until they released their hemolymph by reflex bleeding from the joint between tibia and femur and elytral edges (adults), or setae (larvae). When this treatment was insufficient, a middle leg was cut with micro-scissors. The emanating hemolymph was absorbed directly from one individual insect onto small disks of sterile filter paper (3 mm diam). No distinction between males and females was made for either adults or larvae. Samples on paper disks were applied directly onto agar plates that contained the microorganisms and their respective growth media. Numbers of replications for treatments of adults were $N>9$, for treatments of larvae $N=12$. Untreated sterile filter paper disks were used as controls.

Bacteria For detection of hemolymph activity or synthetic compounds against bacteria, test plates were prepared as described by Gross et al. (1998, 2002).

Yeast Antifungal activity was determined with an inhibition zone assay against live *S. cerevisiae* according to Gross et al. (1998). The yeast was grown for 24 hr at 37°C in 100 ml of Sabouraud broth (Roth). Inhibition zone assays were performed on Sabouraud agar (Roth). After sterilization, the medium was mixed with 2 ml of yeast suspension from the submerged culture. Each Petri dish was filled with 5 ml of the inoculated medium.

Headspace The antimicrobial activity of eleven synthetic volatile organic compounds (VOCs) known from the headspace of adult *H. axyridis* (Cai et al., 2007) were tested against three model microorganisms (*E. coli, M. luteus, S. cerevisiae*) as described above: benzaldehyde (Aldrich), phenol (Roth), (+)-α-pinene (Fluka), (−)-α-pinene (Fluka), n-heptane (Merck), n-octane (Merck), n-nonane (Merck), toluol (Roth), R-(+)-limonene (Aldrich), 2-butanone (Fluka), 2-pentanone (Fluka). Two μl of undiluted VOCs were pipetted into a hole (3 mm diam) punched previously into the agar. Each test was replicated ten times.

Evaluation of Antibacterial and Antifungal Activity After application of the hemolymph samples, Petri dishes were kept at 37°C for 24 hr. Diameters of inhibition zones caused by the samples through radial diffusion into the agar were measured after 24 hr. Series of similar inhibition zone tests were conducted using different concentrations of the antibiotic gentamicin (Serva) for all bacteria or nystatin (Serva) for yeast. The straight calibration lines obtained were used to calculate the antimicrobial activity in μg/ml of the respective antibiotic.

Activity Determination for Key Enzymes

Measuring of Enzyme Activity Muramidase (lysozyme-like) activity against cell walls of Gram-positive bacteria was measured by using the lytic zone assay with freeze-dried *M. luteus* (Sigma, Deisenhofen, Germany) as described previously (Gross et al., 2002). Some of the hemolymph samples caused clear zones in the turbid agar by dissolving the bacterial cell walls. Diameters of these lytic zones were measured after 24 hr of incubation at 37°C. Units/ml lysozyme were calculated using dilutions of chicken lysozyme (Sigma).

Determination of the Minimal Inhibitory Concentration (MIC)

Hemolymph Collection Hemolymph was collected from unchallenged adult beetles. The number of replications for each test group was >5. One hind leg was cut with microscissors directly above the femur from live and dead insects. The secreted hemolymph was absorbed immediately with a glass microcapillary pipette (Hirschmann ringcaps, max. vol. 5 μl). The collected hemolymph was diluted in water (micropore quality) in a sterile Eppendorf tube. The hemolymph solution was centrifuged at 10,000 rpm for 1 min. The supernatant was transferred to a fresh Eppendorf tube and diluted 1:20 with sterile water. This solution then was used directly in growth inhibition assays (see below).

Assays to Determine the Minimal Inhibitory Concentration (MIC) of the Hemolymph The antimicrobial activity of hemolymph from live and dead beetles was evaluated with broth microdilution assays. Bacteria and yeast were grown overnight in liquid media with shaking at 28°C. Prior to experiments an inoculum of bacteria was transferred to liquid Mueller Hinton Broth (MHB) (Fluka), while the yeast strain was cultivated in Sabouraud Dextrose Broth (Roth). Microbial growth was monitored as absorbance of the overnight cultures at 600 nm. Aliquots were removed and plated on the respective nutrient agar in order to determine the numbers of CFU at 28°C. The optical density (OD) of each culture was determined, and cultures were diluted to a final concentration of 10^6 CFU. Ninety-six well flat bottom microtiter plates (Sarstedt) were used in the inhibition assays. The cultures were serially diluted. Forty μl hemolymph solution at a concentration of 5 μl/ml medium were pipetted into the first well and then 1:2 diluted with each of the following pipetting steps until the 12th well in each row. In each assay, the inhibitory effect of hemolymph from live and dead beetles was compared for the organism tested. Gentamicin at a concentration of 50 μg/ml was used as positive reference. In the first well, 20 μg/ml gentamicin were applied and 1:2 diluted until the 12th well. For MIC tests with *S. cerevisiae*, 50 μg nystatin per ml dimethylsulfoxid (DMSO) were used as reference. Aliquots of 100 μl of the bacterial and fungal cultures were added to each well. All microorganisms were tested separately. All tests were repeated five times with three rows from the same hemolymph pool each time. Microtiter plates were incubated in a shaker for 22 hr at 28°C. Antimicrobial activity was assessed by visually detecting turbidity in the wells. Growth inhibition was verified with a spectrophotometer at 600 nm (Microplate reader Floustar Omega, BMG Labtech). Data were collected with the software Omega 1.10 (BMG Labtech) and analyzed (Mars 1.11, BMG Labtech). The MIC was defined as the lowest concentration of the hemolymph or the antibiotic that inhibited all visually detectable microbial growth.

Statistical Analysis Differences in the antimicrobial activity of hemolymph from adults and larvae of the two ladybird species *C. septempunctata* and *H. axyridis*, as well as their

lysozyme-like activity were analyzed by Mann-Whitney *U* tests (Sachs, 1992). All statistical tests were performed with Statistica 5.5 software (StatSoft, 1999).

Results

Antimicrobial Activity The hemolymph of both adults and larvae of *C. septempunctata* showed a weak activity against Gram-positive bacteria (*M. luteus*), nearly no activity against Gram-negative bacteria (*E. coli*), and no activity against yeast (*S. cerevisiae*) (Table 1). While injection of sterile water increased the antibacterial activity in adults, only the injection of a dense bacterial suspension induced an increase in activity against Gram-negative *E. coli* in larvae. The activity against Gram-positive *M. luteus* increased slightly in larval hemolymph after water injection, but increased significantly when dead bacteria were injected ($P<0.05$, Mann-Whitney *U* test). In contrast, the hemolymph of *H. axyridis* strongly inhibited the growth of both bacteria and yeast (Table 1). Inhibition was strongest against *E. coli*, followed by *M. luteus*, and lowest against yeast. Compared to *C. septempunctata*, the antibacterial activity in *H. axyridis* hemolymph was between 640 (Gram-positive bacteria) and up to 16,000 times (Gram-negative bacteria) higher. These results were obtained irrespective of whether the ladybirds had been untreated or immune challenged ($P>0.05$, Mann-Whitney *U* test). The activity against yeast decreased after injection of water or an inactivated bacteria suspension into larvae, and after injection of bacteria in adults compared to untreated control ($P>0.05$, Mann-Whitney *U* test).

Minimal Inhibitory Concentration (MIC) The hemolymph of live *H. axyridis* inhibited the growth of yeast and all three bacteria tested. Inhibition was strongest for *E. coli* and *S. cerevisiae*, followed by *B. subtilis*, and lowest for *B. thuringiensis* subsp. *tenebrionis*. This species also was least susceptible to gentamicin (Table 2). After freezing the beetles at −20°C for a period of 13 to 66 days prior to hemolymph collection, the inhibitory activity decreased significantly, but the same susceptibility pattern was observed (Table 2). The concentration of gentamicin necessary to fully inhibit growth of *E. coli* was 0.2 μg/ml. The amount of hemolymph from live beetles (0.16 μl/ml) necessary for similar growth inhibition of *E. coli* was about four times lower than the hemolymph amount necessary to fully inhibit *B. subtilis* (0.63 μl/ml), and 2.5 μl/ml were necessary to inhibit the growth of *B. t. tenebrionis* (Table 2). The inhibitory effect of hemolymph from frozen beetles was eight times lower for *E. coli*, eight times lower for *B. subtilis*, and 2 times lower for *B. t. tenebrionis* compared to hemolymph from live beetles. The concentration of antifungal nystatin necessary to obtain complete growth inhibition of yeast was 3.13 μg/ml. A similar growth inhibition was observed by adding 0.16 μl hemolymph from live beetles to the medium. Its antifungal activity was four times higher compared to hemolymph from frozen beetles (Table 2).

Enzyme Activity Enzyme activity in the hemolymph of *H. axyridis* was higher than in *C. septempunctata* (Fig. 1a, b). In larvae of both species, hemolymph activity was higher than in adults. We observed an increase in the lysozyme-like activity in treated larvae (water, bacteria) compared to untreated larvae within both species (Mann-Whitney *U* test, $N=12$, $P<0.001$). There was no statistically significant difference between untreated and treated adults within either species ($P>0.05$, Mann-Whitney *U* test). Differences between treatments were not statistically significant ($P>0.05$, Mann-Whitney *U* test).

Table 1 Mean values and standard deviations of antimicrobial activity of hemolymph of two ladybird species *Coccinella septempunctata* and *Harmonia axyridis* in μg/ml antibiotic equivalent (gentamycin: *Escherichia coli* and *Micrococcus luteus*; nystatin: *Saccharomyces cerevisiae*) analyzed by agar diffusion assay

Stage	Microbe	*C. septempunctata*						*H. axyridis*					
		Untreated		Water		Bacteria		Untreated		Water		Bacteria	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Larva	*E. coli*	0^{a}	0	0^{a}	0	9^{b}	9	9064^{a}	4654	16460^{a}	9192	15249^{a}	14348
	M. luteus	0^{a}	0	41^{b}	94	116^{c}	63	6376^{a}	2585	8618^{a}	4365	8723^{a}	4592
	S. cerevisiae	0^{a}	0	0^{a}	0	0^{a}	0	223^{a}	86	131^{b}	60	110^{b}	41
Adult	*E. coli*	0^{a}	0	6^{b}	7	4^{b}	3	8818^{ab}	4021	6004^{a}	4070	11925^{b}	6807
	M. luteus	0^{a}	0	24^{b}	33	28^{ab}	41	23803^{a}	8941	21401^{a}	7786	18548^{a}	10291
	S. cerevisiae	0^{a}	0	0^{a}	0	0^{a}	0	1963^{a}	721	1681^{ab}	750	1090^{b}	571

Numbers of replications for treatments of adults are >9, for treatments of larvae are 12. Different letters indicate significant differences within treatments (*Mann-Whitney U test*, $P<0.05$)

Table 2 Minimal inhibitory concentrations (MIC) of hemolymph of live and frozen adult *Harmonia axyridis* (μl/ml) and antibacterial (gentamycin) or antifungal (nystatin) standards (μg/ml)

Agent	*E. coli*	*B. subtilis*	*B. thuringiensis*	*S. cerevisiae*
live	0.16	0.63	2.50	0.16
frozen	1.25	5.00	>5.00	0.63
Gentamycin	0.20	0.20	3.13	–
Nystatin	–	–	–	3.13

The number of replications for each group is >5

Headspace Three out of eleven tested VOCs known from the headspace of *H. axyridis* (phenol, (+)-α-pinene, benzaldehyde) inhibited the growth of bacteria, *E. coli* and *M. luteus*, as well as the yeast (Fig. 2). The other VOCs tested showed no inhibitory effects.

Discussion

The hemolymph from unchallenged *H. axyridis* strongly inhibited the growth of all tested microorganisms. By contrast, the hemolymph from unchallenged *C. septempunctata* showed no inhibitory effects against the same microorganisms. After injection of water or inactivated bacteria, the antibacterial activity in the hemolymph of *C. septempunctata* adults and larvae increased significantly. It is striking that neither treatment challenged the immune system of *H. axyridis*, but at the same time decreased the antifungal activity in its hemolymph. To date no other study has reported similar findings. This leads us to suggest a possible interference in the induction of different immune pathways. It was reported earlier (Lemaitre et al., 1997) that insects might respond to different challenges and, thus, adapt their disease resistance accordingly while

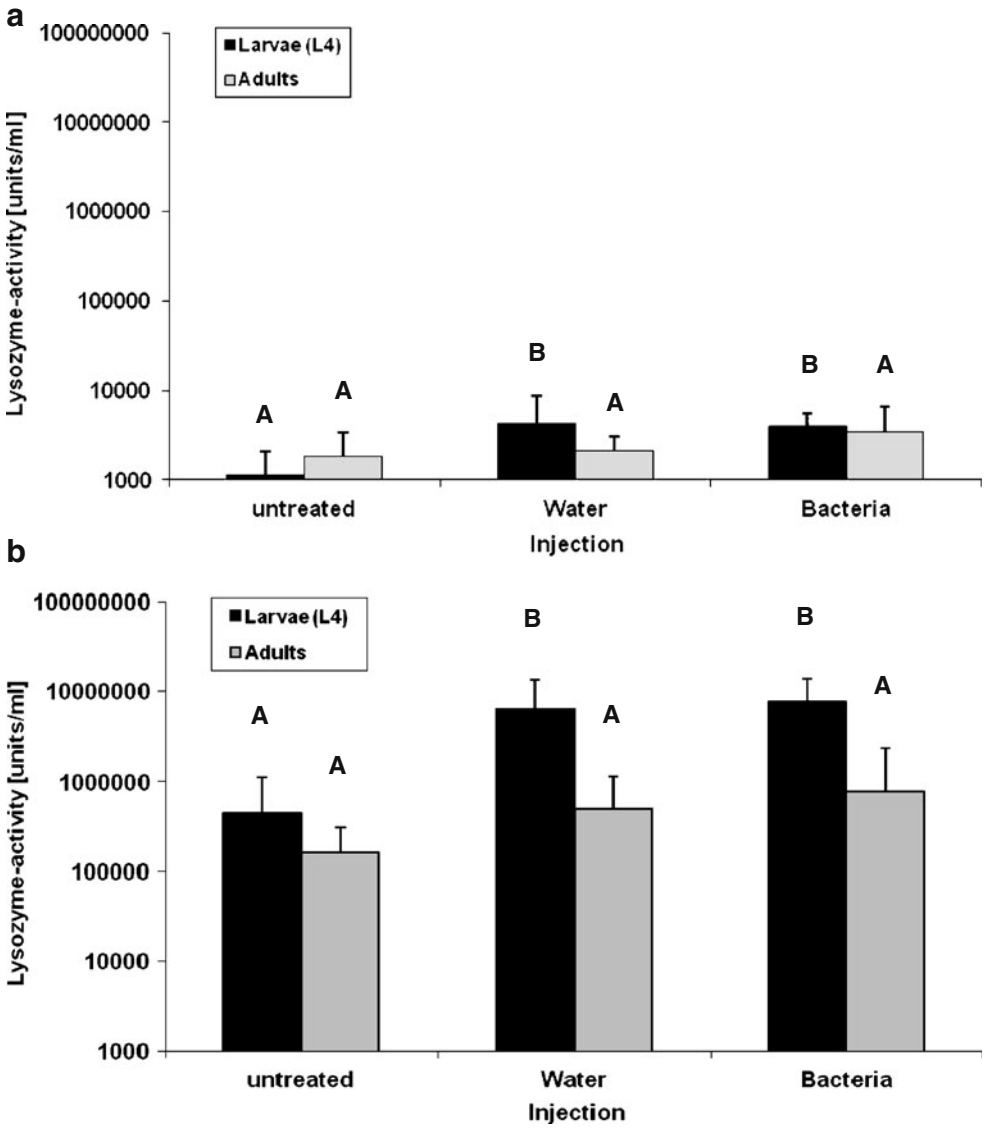

Fig. 1 Lysozyme activity [units/ml] of the hemolymph of ladybird beetles. **a**: *Coccinella septempunctata*. **b**: *Harmonia axyridis*. While there is no statistically significant difference between the three treatments of adults of both species (*grey bars*), there is an increase in the lysozyme-like activity in treated larvae (water, bacteria) compared to untreated larvae within both species (*black bars*; Mann-Whitney *U* test, $N=12$, $P<0.001$)

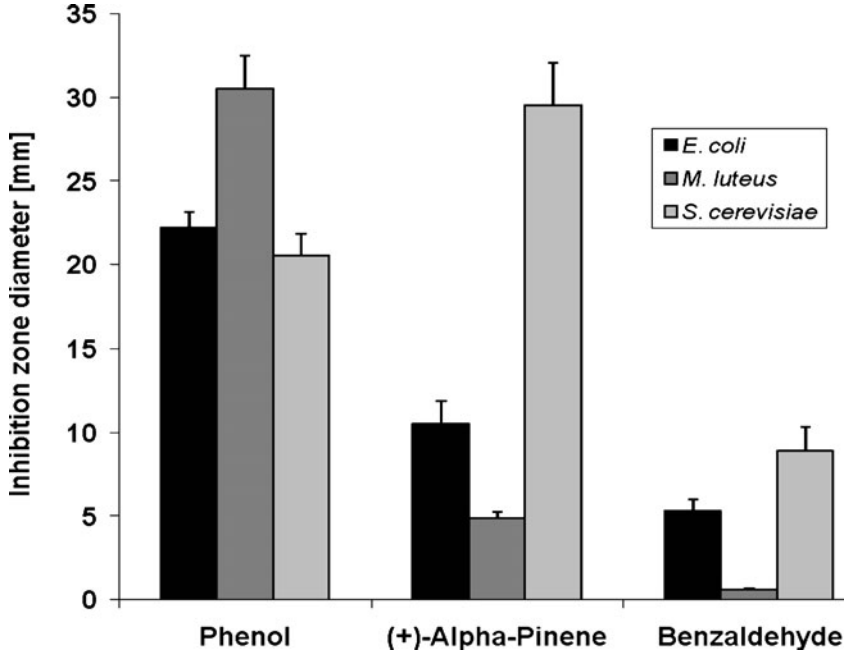

Fig. 2 Growth inhibition of bacteria (*Escherichia coli, Micrococcus luteus*) and yeast (*Saccharomyces cerevisiae*) by synthetic VOCs (phenol, (+)-α-pinene, benzaldehyde) present in the headspace of *Harmonia axyridis*. *Bars* represent mean and standard deviation of inhibition zone diameters (mm, $N=10$)

minimizing the resources necessary for this defensive response.

Lysozyme activity against *M. luteus* was more than hundredfold higher in the harlequin ladybird when compared to *C. septempunctata*. Induction increased the lytic activity in both larvae but not in adults of either species (Fig. 1). The higher activity of this enzyme might assure increased survival rates of larvae in the populations of the invasive *H. axyridis* compared to *C. septempunctata*. Furthermore, adults of *H. axyridis* additionally produce 38 volatile organic compounds (VOCs) (Cai et al., 2007). Three of them, phenol, (+)-α-pinene, and benzaldehyde, inhibited the growth of both bacteria and yeast in our assays. Additionally, a synergistic amplification of antimicrobial active VOCs is possible. These compounds may engulf the insects with an antimicrobial cloud. This phenomenon was recently reported for leaf beetles (Gross et al., 2008; Gross and Schmidtberg, 2009). Such a mechanism could be important, especially during overwintering, as *H. axyridis* forms aggregations consisting of hundreds of individuals in cavities under rocks or plants. Further studies will be necessary to test this hypothesis.

The MIC assays showed, that hemolymph of untreated adult *H. axyridis* inhibited the growth of Gram-negative *E. coli* and *S. cerevisiae* more strongly than the growth of Gram-positive bacteria (*B. subtilis* and *B. t. tenebrionis*). Moreover, the hemolymph from live beetles was significantly more efficient than hemolymph from frozen beetles against all organisms tested. We observed a difference in the susceptibility of *S. cerevisiae* between these two assays. The yeast was more susceptible to hemolymph in the liquid medium MIC assays compared to agar diffusion assays. This could be due to a better contact of the active compounds in the fluid than on the agar.

Antimicrobial peptides (AMP) effective against Gram-negative organisms known from invertebrates generally are produced after recognition of foreign compounds. They are, thus, considered induced defenses. We observed the contrary in *H. axyridis*; the hemolymph of unchallenged beetles caused in agar diffusion assays and MIC tests strong inhibition of microbial growth. This finding suggests high concentrations of constitutive antimicrobial compounds present in hemolymph. Inducible AMPs have been isolated from several insect orders, but few studies have been conducted with Coleoptera (Bulet et al., 1991). The chemical nature of the antimicrobial compounds in the hemolymph of *H. axyridis* is not yet known. The costs of maintaining a highly efficient immune system have been related to an inferior tolerance of starvation and desiccation in parasitoid resistant *Drosophila* (Hoang, 2001). Few data are available on stress tolerance of *H. axyridis* (Agarwala et al., 2008) and nothing is known how stress affects its immune system. We currently are conducting studies to evaluate various stress factors and their effects on the antimicrobial defense of *H. axyridis*.

Interestingly, in microtiter plate assays, we observed that the hemolymph of *H. axyridis* partially lost its activity after freezing live beetles at −20°C prior to hemolymph extraction. This effect was strongest against Gram-positive bacteria (*Bacillus subtilis, B. thuringiensis*) and weaker against Gram-negative *E. coli* and yeast (Table 2). A destruction of the antimicrobial compound due to the death

of the beetles is unlikely because the freezing of live beetles should stop immediately any destructive physiological processes. In an earlier study, it was suggested that low temperatures may change the qualitative and quantitative composition of antimicrobial peptides in insect hemolymph (Stephen and Johnson, 1962). The observed changes were, however, variable between species and age groups of the tested cockroaches. Duration of freezing had no effect on protein composition. Stephen and Johnson (1962) found qualitative changes of hemolymph proteins more pronounced for weak clot producing cockroach species. The hemolymph of multicolored lady beetles produces such clots only very slowly upon secretion (pers. observ.). Corresponding to Stephen's observations, protein stability is not assured after freezing and thawing of hemolymph samples. The reduction of the antimicrobial efficacy observed in our studies might be due to thawing of the frozen beetles prior to hemolymph collection. To our knowledge, no other studies have reported a similar loss of the inhibitory effect, since neither hemolymph from live nor from dead insects has been evaluated. Further assays on the kinetic of microorganism growth are necessary to verify the bactericidal/fungicidal or merely growth inhibitory effect of the hemolymph observed in our assays.

Gram-negative *E. coli* and the yeast *S. cerevisiae* were more susceptible to the antimicrobial substances in the hemolymph of adult *H. axyridis* than the two Gram-positive *Bacillus* species. In contrast, the third Gram-positive species tested, *M. luteus*, also was strongly inhibited. It has been reported that *M. luteus* is highly sensitive to chicken lysozyme (Altincicek et al., 2008). The high lysozyme activity in the hemolymph of *H. axyridis* compared to *C. septempunctata* may explain the strong inhibition of bacteria by both adult and larval hemolymph. It is not surprising that higher concentrations of antimicrobial compounds were necessary to inhibit the growth of insect pathogenic *B. t. tenebrionis*. This strain has been isolated from a beetle (*Tenebrio molitor*) and is mainly infective for beetles (Krieg et al., 1984). Recently, it was reported that the antimicrobial peptide rhinocerosin isolated from the coconut rhinoceros beetle is more active against Gram-positive bacteria with a MIC value of 1.0 μg/ml (Yang et al., 1998). A study on two different synthetic antimicrobial peptides found a wide variation in MIC values of 128 μg/ml, 16 μg/ml, and 4 μg/ml against *E. coli* (Kamysz and Turecka, 2005). For *H. axyridis*, it is not yet known whether antimicrobial proteins or other factors (review in Boman and Hultmark, 1987) are responsible for the observed microbial growth inhibition.

The differences in the antifungal activity between the two ladybird species tested are notable. Whereas the hemolymph from untreated adult *C. septempunctata* had no antifungal activity, the inhibitory activity of hemolymph from adult *H. axyridis* beetles corresponded to 100–1,000 μg/ml of the antimycotic nystatin. Little is known about the antifungal activity of insect hemolymph, probably because of a low search effort for antifungal proteins (Bulet and Stöcklin, 2005). Few studies, however, have reported strong growth inhibition of fungi by inducible insect hemolymph compounds (Lamberty et al., 1999), and defensins and cecropins are known for their antibacterial but also antifungal activities (Vilcinskas and Gross, 2005). Hence, our study is the first to report antifungal activity in an unchallenged beetle species. The presence and rapid response of the constitutive defenses in *H. axyridis* might be another factor that could help explain the competitive superiority of this exotic species when compared to native ladybird beetles.

In the present study, we did not differentiate between sexes or morphs with different elytral colors. Recent preliminary data suggest no differences in disease resistance between female and male *H. axyridis* (Gross, unpubl. data). Further studies of variations in hemolymph activity in different life stages, sexes, and morphs are necessary to understand its importance for the competitive success of *H. axyridis* (Fedorka et al., 2004).

Most studies have focused on behavioral differences between *H. axyridis* and native ladybirds in Europe (Soares and Serpa, 2007; Brown et al., 2008), Canada (Labrie et al., 2006), the USA (Yasuda et al., 2004) and Japan (Ware and Majerus, 2008). Larger size and physically better defended larval stages contribute to higher survival rates (Snyder et al., 2004) and to superiority in aggressive encounters with other ladybird species (Pell et al., 2008). Cannibalism and intraguild predation have been observed even when aphid prey is abundant (Pell et al., 2008). In Canada, *H. axyridis* has a shorter development time for younger larval stages than indigenous ladybird species (Labrie et al., 2006). Furthermore, *H. axyridis* adults are rarely preyed upon (Pell et al., 2008), and under laboratory conditions, adult beetles are rejected by the predatory hemipteran, *Podisus maculiventris* (Houg-Goldstein et al., 1996; DeClercq et al., 2003). Cottrell and Shapiro-Illan (2003, 2008) found that *H. axyridis* is more resistant to various *B. bassiana* strains when compared to highly susceptible native coccinellids. Roy et al. (2008) showed for three species of ladybirds that much higher concentrations of *Beauveria bassiana* conidia were required to artificially infect *H. axyridis*.

In summary, the observed disease resistance against antagonistic microorganisms is one more fitness parameter that contributes to the enormous capacity of *H. axyridis* to exploit new habitats and to out-compete other coccinellid species. The importance of disease resistance for life history traits and population dynamics of *H. axyridis* under different climatic and geographic conditions awaits further studies.

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