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Invasive Harlequin Ladybird Carries Biological Weapons Against Native Competitors

Andreas Vilcinskas,[1,3]*† Kilian Stoecker,[2,3]† Henrike Schmidtberg,[3] Christian R. Röhrich,[3] Heiko Vogel[4]

Invasive species that proliferate after colonizing new habitats have a negative environmental and economic impact. The reason why some species become successful invaders, whereas others, even closely related species, remain noninvasive is often unclear. The harlequin ladybird *Harmonia axyridis*, introduced for biological pest control, has become an invader that is outcompeting indigenous ladybird species in many countries. Here, we show that *Harmonia* carries abundant spores of obligate parasitic microsporidia closely related to *Nosema thompsoni*. These microsporidia, while not harming the carrier *Harmonia*, are lethal pathogens for the native ladybird *Coccinella septempunctata*. We propose that intraguild predation, representing a major selective force among competing ladybird species, causes the infection and ultimate death of native ladybirds when they feed on microsporidia-contaminated *Harmonia* eggs or larvae.

Human activities, particularly international trade, promote the spread of invasive species that cause extensive economic losses and negatively affect native species. Several factors can play a role in the invasive success of such species, including the lack of predators, short generation times, and the ability to disperse rapidly and adapt easily to new habitats (*1*, *2*). However, the principles that allow some species to become successful invaders, whereas most (even if closely related) do not, remain poorly understood (*3*). Invaders can be released from native, coevolved pathogens, but they face other pathogens in their new environments, suggesting that the ability to mount strong antimicrobial defenses may promote invasive success (*4*). Yet enhanced immunity can be costly and, therefore, can be traded off against other traits such as growth and reproduction (*5*, *6*). We used the harlequin ladybird *Harmonia axyridis* (a native species in central Asia) as a model to explore the potential role of immunity in invasion biology. This species has been introduced into many countries as a biological control agent against aphids and other insect pests but is now causing severe problems because it successfully outcompetes native ladybird species in many areas (*7*).

We recently showed that, in contrast to native ladybird species in Europe, the *Harmonia* hemolymph contains strong and constitutive antibacterial activity throughout development. We attributed this activity to harmonine, a secondary metabolite that accumulates to high levels in the hemolymph. The broad-spectrum activity of this alkaloid compound is demonstrated by its ability to inhibit even human pathogens such as *Mycobacterium tuberculosis* and *Plasmodium falciparum*, making it a promising lead for the development of new anti-infective drugs (*8*). Constitutive harmonine activity may help *Harmonia* to deal with pathogens encountered in new habitats, whereas native ladybirds are more susceptible to infection (*9*).

Independently, harmonine has been proposed as a chemical defense compound protecting *Harmonia* eggs and larvae from predation by native ladybird species (*10*). Kajita *et al.* reported that

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[1]Institute of Phytopathology and Applied Zoology, Heinrich-Buff-Ring 26-32, Justus-Liebig-University of Giessen, D-35392 Giessen, Germany. [2]Bundeswehr Institute of Microbiology, Neuherbergstrasse 11, D-80937 Munich, Germany. [3]Fraunhofer Institute of Molecular Biology and Applied Ecology, Winchesterstrasse 2, 35394 Giessen, Germany. [4]Max-Planck-Institute of Chemical Ecology, Hans-Knoell-Strasse 8, D-07745 Jena, Germany.

*Corresponding author. E-mail: andreas.vilcinskas@agrar.uni-giessen.de

†These authors contributed equally to this work.

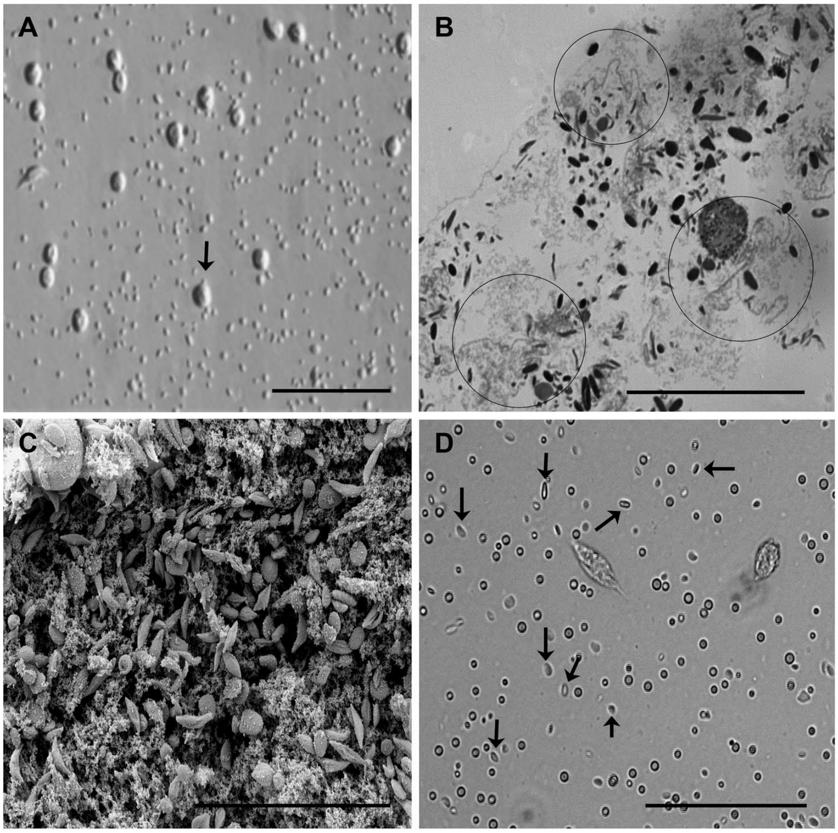

Fig. 1. Microscopy studies of microsporidia. (**A**) Light microscopy image showing the high concentration of microsporidia (small objects) between the larger hemocytes (arrow) in *Harmonia* hemolymph. Scale bar, 50 μm. (**B**) Semithin sections of hemolymph tissue confirm the presence of microsporidia, among which some exhibit extruded polar tubes (circles). Extrusion of polar tubes occurred during fixation. Scale bar, 20 μm. (**C**) Scanning electron microscopy image of microsporidia in the *Harmonia* hemolymph, showing the high load of spores. Scale bar, 20 μm. (**D**) Light microscopy image showing microsporidia (arrows) between yeastlike cells and larger hemocytes in dying *Coccinella* beetles 7 days postinoculation. Scale bar, 20 μm.

the ingestion of *Harmonia* eggs by native *Coccinella septempunctata* beetles caused mortality, but the reciprocal situation was nonlethal. In agreement with our previous report (*8*), they detected high concentrations of harmonine in *Harmonia* eggs and concluded that this compound protects the invasive ladybird from intraguild predation (*10*), which is a major selective force among competing ladybird species (*11*).

Here, we report that the injection of *Harmonia* hemolymph—but not synthetic harmonine alone, even in high concentrations (fig. S1)—can kill *Coccinella* beetles, making it unlikely that the mortality caused by feeding on *Harmonia* eggs is caused by the presence of harmonine. It is therefore apparent that the mortality in *Coccinella* beetles is caused by another component found in the hemolymph of *Harmonia*.

Light microscopy revealed the presence of abundant microsporidia among the hemocytes in *Harmonia* hemolymph (Fig. 1A) (see supplementary materials and methods). Microsporidia are obligate parasites that replicate within eukaryotic cells after penetrating the plasma membrane with an extruded polar tube. Semithin sections of hemolymph tissue confirmed the presence of microsporidia, among which some exhibited extruded polar tubes (Fig. 1B). We used scanning electron microscopy to document the high abundance of microsporidia in the hemolymph of *Harmonia* (Fig. 1C). Despite the abundance of spores, we did not find any *Harmonia* beetles that were killed by the parasites in our rearing. The low physiological activity of the microsporidia is further supported by the absence of microsporidial gene expression when analyzing the transcriptome of *Harmonia* eggs and beetles (*12*). These data suggest that the microsporidia are present in a physiologically inactive spore stage and do not harm their host, perhaps because it has acquired tolerance or resistance.

We identified the microsporidia by amplifying the small-subunit ribosomal RNA (rRNA) genes using a variety of previously described primer sets (*13*), resulting in the specific amplification of the partial microsporidial 16*S* rRNA gene. The determined sequence identity (≥99%) placed the *Harmonia*-associated microsporidia within the *Nosema*/*Vairimorpha* clade, with *Nosema thomsoni* as the closest relative (*14*). This assay confirmed the presence of *Nosema*-like microsporidia in all *Harmonia* beetles of all populations sampled, as well as in eggs and larvae, suggesting vertical transmission. All attempts to coculture the microsporidia with different insect cell lines (Sf9, Sf21, *Drosophila* S2, High Five) failed.

Given that (i) *Coccinella* beetles are killed by feeding on *Harmonia* eggs and larvae, but the reciprocal situation is not lethal (*10*), and (ii) harmonine does not affect *Coccinella* beetles, even at high concentrations, we propose that native ladybird species may be lethally infected by the microsporidia carried by *Harmonia* when they feed on its eggs and larvae. To test this hypothesis, we collected hemolymph samples from *Harmonia* and isolated the microsporidia by repeated centrifugation and washing steps. We divided the purified microsporidia into two portions, one of which was heat-inactivated and used for control injections.

All *Coccinella* beetles injected with live microsporidia isolated from *Harmonia* died within 2 weeks (Fig. 2), whereas the majority of control beetles injected with either the heat-inactivated microsporidia or the buffer alone survived. Control injections with cell-free hemolymph samples from *Harmonia* lacking microsporidia and hemocytes did not result in enhanced mortality (fig. S4). This observation and the analysis of injected samples by SDS–polyacrylamide gel electrophoresis (fig. S2) and mass spectrometry ruled out the possibility that the mortality was caused by thermolabile toxins in the hemolymph of *Harmonia*. Further, we determined the presence of microsporidia in dying *Coccinella*, but not in control beetles (Fig. 1D and fig. S3). We concluded that the microsporidia carried by *Harmonia* were lethal but required some time to infect and replicate within *Coccinella*.

The high abundance of tolerated microsporidia in *Harmonia* hemolymph and their ability to kill *Coccinella* beetles support our hypothesis that these parasites contribute to the dominance of *Harmonia* over native species. Obviously, native ladybirds such as *Coccinella* do not share with *Harmonia* the ability to suppress microsporidial replication. It remains to be seen whether or not harmonine (*8*) and/or the tremendous spectrum of antimicrobial peptides discovered in *Harmonia* (*12*) contribute to its tolerance or resistance against microsporidia. Our data also provide a candidate mechanism to explain why the decline in native ladybird numbers is associated with intraguild predation (*11*). The presence of microsporidia in *Harmonia* may function like a biological weapon, in accordance with the novel weapons theory (*15*).

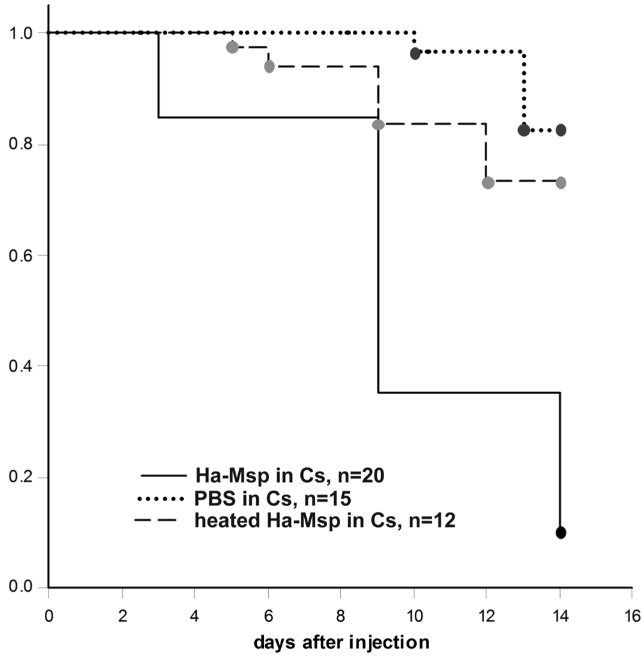

Fig. 2. Survival after injection. *Coccinella* beetle survival rate (*y* axis) analysis after transfer of microsporidia isolated from the hemolymph of *Harmonia* (Ha-Msp in Cs), calculated by Kaplan Meier survival analysis log-rank test. *Coccinella* beetles were injected with living microsporidia (solid line). Control injections with either heat-inactivated microsporidia (heated Ha-Msp) isolated from *Harmonia* (dashed line) or PBS alone (dotted line) showed statistically significant differences ($P < 0.05$), indicating that mortality of *Coccinella* is predominantly caused by living microsporidia. Circles indicate where animals have been taken out of the experiment to control the presence of microsporidia in the hemolymph. Cs, *Coccinella septempunctata*; PBS, phosphate-buffered saline.

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Supplementary Materials
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Materials and Methods
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