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Eating chemically defended prey: alkaloid metabolism in an invasive ladybird predator of other ladybirds (Coleoptera: Coccinellidae)

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SUMMARY

By comparison with studies of herbivore physiological adaptation to plant allelochemicals, work on predator physiological adaptation to potentially toxic prey has been very limited. Such studies are important in understanding how evolution could shape predator diets. An interesting question is the specificity of predator adaptation to prey allelochemicals, given that many predators consume diverse prey with different chemical defences. The ladybird *Harmonia axyridis*, an invasive species in America, Europe and Africa, is considered a significant predatory threat to native invertebrates, particularly other aphid-eating ladybirds of which it is a strong intraguild predator. Although ladybirds possess species-specific alkaloid defences, *H. axyridis* exhibits high tolerance for allospecific ladybird prey alkaloids. Nonetheless, it performs poorly on species with novel alkaloids not commonly occurring within its natural range. We examined alkaloid fate in *H. axyridis* larvae after consumption of two other ladybird species, one containing an alkaloid historically occurring within the predator's native range (isopropyleine) and one containing a novel alkaloid that does not (adaline). Our results indicate that *H. axyridis* rapidly chemically modifies the alkaloid to which it has been historically exposed to render it less harmful: this probably occurs outside of the gut. The novel, more toxic alkaloid persists in the body unchanged for longer. Our results suggest metabolic alkaloid specialisation, in spite of the diversity of chemically defended prey that the predator consumes. Physiological adaptations appear to have made *H. axyridis* a successful predator of other ladybirds; however, limitations are imposed by its physiology when it eats prey with novel alkaloids.

Key words: alkaloids, Coccinellidae, *Harmonia axyridis*, intraguild predation, metabolism, physiological tolerance.

INTRODUCTION

Many predators consume at least a proportion of chemically defended prey in their diet (e.g. Reitze and Nentwig, 1991; Morrison et al., 2007). However, there are relatively few well-studied examples of predator physiological adaptation to such prey (e.g. Geffeney et al., 2002; Hartmann et al., 2003). This stands in contrast to the large body of work on the adaptations of herbivores to plant chemical defences (Brattsten, 1992; Li et al., 2007). Because most predators consume a diversity of prey, it is expected that predator metabolism will be adapted to deal with a broad range of potential toxins, as appears to be the case for generalist herbivores (Li et al., 2004); however, the specificity of predator allelochemical metabolism remains largely untested. A better knowledge of the extent and nature of predator physiological tolerance of prey allelochemicals is important in understanding how evolution can shape predator diets and prey choice (Pekár and Toft, 2009), as well as providing an insight into tolerance of other xenobiotics, such as insecticides and pollutants (Li et al., 2007).

A particularly suitable model for studying predator physiological adaptation to chemical defence is intraguild predation (IGP) among aphidophagous ladybird beetles, i.e. ladybirds eating each other in addition to aphid prey (Lucas, 2005). Ladybirds are defended by autogenously produced alkaloids, which are interspecifically variable and generally persist through all stages of the life cycle (Pasteels et al., 1973; Ayer and Browne, 1977; Daloze et al., 1995; King and Meinwald, 1996). When a ladybird eats another ladybird species, with different alkaloid defences, it frequently suffers toxic effects (Hemptinne et al., 2000a; Cottrell, 2004), and many species utilise egg-surface hydrocarbons to avoid interspecific prey, unless starving (Hemptinne et al., 2000b; Omkar et al., 2004). Nonetheless, IGP does occur between ladybird species and a few species appear to be adapted to attack and eat other species of ladybird (Lucas, 2005; Sloggett et al., 2009a).

The most notorious ladybird intraguild predator is *Harmonia axyridis* (Pallas), an Asian native that is invasive in North and South America, Western and Central Europe and Southern Africa (Koch et al., 2006; Stals and Prinsloo, 2007; Brown et al., 2008). A particular reason for its notoriety is the threat it is believed to pose through the predation of other invertebrates, particularly other ladybirds, of which it is considered a habitual predator (Koch and Galvan, 2008; Pell et al., 2008). Unlike most other ladybirds, *H. axyridis* exhibits a high tolerance for other species' alkaloids (e.g. Sato and Dixon, 2004; Sato et al., 2008). Nonetheless, this adaptation is limited to the alkaloids possessed by ladybird species with which *H. axyridis* frequently interacts in its native range. These are generally palatable to *H. axyridis*, and may exhibit behavioural adaptations to limit *H. axyridis* IGP (Ware et al., 2008; Ware and Majerus, 2008). *Harmonia axyridis* preys indiscriminately upon a diversity of ladybirds where it is invasive. However, it performs significantly less well on species with novel alkaloids, defined as those alkaloids not occurring in species within its native range or occurring in species with which *H. axyridis* rarely interacts, i.e. those species with which it shares little or no coevolutionary history (Sloggett et al., 2009a).

Although a vast number of studies have examined *H. axyridis* feeding ecology in the context of IGP and the threat it poses to

biodiversity, little is known about the physiology of its alkaloid tolerance. In this paper describe a prey alkaloid ADME (absorption, distribution, metabolism and excretion) study of *H. axyridis* intraguild predation, using gas chromatography-mass spectrometry (GC-MS) of prey alkaloids, a method previously used to detect IGP of other species of ladybirds by *H. axyridis* (Hautier et al., 2008; Sloggett et al., 2009b). We compare the fate of an alkaloid from a prey occurring in the native range of *H. axyridis*, on which it performs well, and a novel alkaloid from a prey to which *H. axyridis* has not historically been exposed and on which it performs less satisfactorily. By comparing differences in the metabolism of the two alkaloids, we are able not only to gain an insight into what makes *H. axyridis* a successful predator of other ladybirds but also to examine whether there are limitations imposed by the metabolic specificity of *H. axyridis* when it consumes unfamiliar prey with novel alkaloids.

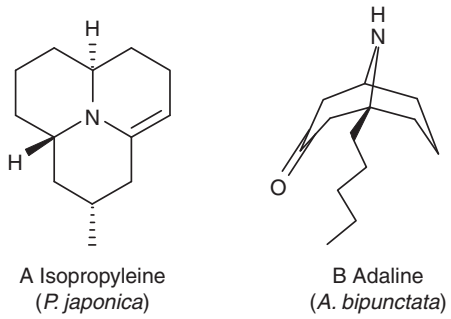

Fig. 1. The structures of the major alkaloids of *Propylea japonica,* isopropyleine, and *Adalia bipunctata*, adaline.

MATERIALS AND METHODS

Ladybird predators and prey

Experiments utilised final (fourth) instar *H. axyridis* larvae preying on eggs of two ladybird species *Propylea japonica* (Thunberg) and *Adalia bipunctata* (L.). Most intraguild predation involves larval predators, and the results from egg consumption are equally applicable to the alkaloids in other life-history stages: their alkaloids are the same and occur in similar concentrations (Daloze et al., 1995). The prey species were chosen because both possess major alkaloids (≥75% of total) that are amenable to GC-MS analysis without complicating analytical factors such as high levels of thermal degradation or the necessity for derivatisation (e.g. Sloggett et al., 2009b). The absolute amount of major alkaloid in the two species, while unlikely to be identical, is expected to be of a similar order of magnitude, because total concentrations of alkaloids in ladybirds are similar across species (Pasteels et al., 1973).

Propylea japonica commonly co-occurs with *H. axyridis* in its native range (e.g. Arefin and Ivliev, 1984; Takahashi and Naito, 1984), and *H. axyridis*, a known intraguild predator of *P. japonica*, develops with negligible mortality on an exclusive diet of larvae of this species in the laboratory (Sato et al., 2008). The alkaloids of *P. japonica*, which were identified for the first time for this work, are two azaphenalenes: isopropyleine (Fig. 1A), the major component, with its double-bond isomer propyleine, the minor component [the two comprising a rapidly interconverting mixture (Mueller and Thompson, 1980)]. *Adalia bipunctata* did not historically occur in the original range of *H. axyridis*. The two species now come into contact in both North America and Europe where *H. axyridis* is invasive (e.g. Harmon et al., 2007; Hautier et al., 2008). They also occur together now in Japan, where *A. bipunctata* has recently become established (Toda and Sakuratani, 2006). *Harmonia axyridis* is a known intraguild predator of *A. bipunctata* in the field (e.g. Toda and Sakuratani, 2006; Hautier et al., 2008), but *H. axyridis* larval mortality on an *A. bipunctata* egg diet is high (Sato and Dixon, 2004). This is best explained as being due to a lack of *H. axyridis* adaptation to *A. bipunctata* alkaloids resulting from a lack of coevolutionary history. The alkaloids of *A. bipunctata*, a major homotropane, adaline (Fig. 1B), and a minor piperidine, adalinine (Tursch et al., 1973; Lognay et al., 1996), are not known from any species that occur in the natural range of *H. axyridis*.

Stocks of *H. axyridis* and *A. bipunctata* originated in Groningen, The Netherlands, while stocks of *P. japonica* originated in Fuchu, Japan. Ladybirds were maintained at, and experiments conducted at, 22±0.5°C and 18 h:6 h light:dark photoperiod. Ladybirds were fed excess pea aphids, *Acyrthosiphon pisum* (Harris) from broad beans, *Vicia faba* (L.), with a small piece of apple (*Malus domestica* Borkhausen) provided as an additional fluid source.

Feeding experiments and collection of samples

Harmonia axyridis larvae were reared from eggs to the fourth instar under the conditions described above. One day after larval moult to the fourth instar, they were starved for six hours, with access only to water, and then provided with three grouped eggs of either *P. japonica* or *A. bipunctata* (cf. Sloggett et al., 2009b). A three-egg meal constitutes a sublethal dose of *A. bipunctata* alkaloid for fourth instars (J.J.S., unpublished data). Eggs were <24 h old and from laboratory-maintained cultures.

Typically the three-egg group was consumed in a single feeding bout. After egg consumption, the larvae were either placed in a freezer immediately at –15°C or returned to the pea aphid diet and frozen 4, 8, 16 or 32 h after egg consumption to provide whole larval bodies for analysis. A second group of larvae were used as a haemolymph source. This was collected at the same time periods by cutting off one larval leg and placing a 0.5 µl microcapillary tube next to the cut surface. Care was taken not to squeeze the larva during this process, to ensure that no gut contents were accidentally released into the haemolymph. The amount of haemolymph collected was quantified by measuring the length of the haemolymph column in the tube, and ranged from 31 nl to 180 nl. Frass pellets were also collected from dishes containing individual larvae 32 h after they had consumed eggs. This is enough time for food to completely pass through the gut (Dixon, 1986). Additionally samples of three eggs from *P. japonica* and *A. bipunctata* eggs were preserved. As with larval bodies, all samples were stored frozen at –15°C.

Sample preparation and GC-MS of samples

All samples were initially extracted in methanol. Three-egg samples and whole predator bodies were homogenized in a 150 µl volume, while frass samples were soaked in a 150 µl volume for 30 min. For haemolymph samples, the microcapillary tubes containing them were crushed into 100 µl methanol. Homogenates were centrifuged at 2683***g*** rpm for 2 min; the methanolic supernatant was then transferred to an autosampler vial [100 µl transferred for initially 150 µl samples (whole bodies, eggs, frass), 80 µl transferred for initially 100 µl samples (haemolymph)]. The methanol was evaporated under a nitrogen stream and the extract was redissolved in 20 µl dichloromethane for GC-MS analysis.

GC-MS analyses used an Agilent 6890 gas chromatograph with an Agilent 7683 autosampler coupled to an Agilent 5973 mass spectrometer (Böblingen, Germany). A split-splitless injector at, 200°C and a Zebron ZB-5 MSi GC column (0.25 mm internal

diameter; 30 m length; 0.25 µm film thickness) were used. The carrier gas was helium with a flow rate of 1 ml min^{-1}. Mass spectra were obtained using electron ionisation mode at 70 eV, scanning was done over the range *m*/*z* 35–400. The GC temperature program was 60°C for 2 min, then a 10°C min^{-1} increase up to 325°C (Sloggett et al., 2009b), with the final temperature held for 3 min. 1 µl of extract was injected.

Alkaloid analysis and quantification

The focus of our analyses was the major prey alkaloids, isopropyleine from *P. japonica* and adaline from *A. bipunctata*. Although both major and minor alkaloids may be toxic, the greatest potential risk to a predator is the more abundant major alkaloid. The alkaloids were identified by comparison of mass spectra from prey extracts with published mass spectra. Isopropyleine retention time was 13.83 min and that of adaline was 16.84 min. Relatively little thermal degradation of adaline was observed in samples (cf. Hautier et al., 2008) and thus thermally degraded adaline was not included in measurements.

Relative quantification of alkaloid in predator samples was derived from peak areas of the most abundant distinctive ion of the alkaloids (176 for isopropyleine, 166 for adaline). This was corrected for the proportion of the original sample injected and, in the case of the haemolymph, the amount collected. Mean ion peak areas obtained from the three-egg samples (*P. japonica*, N=6; A. *bipunctata*, N=7) were used to estimate the relative amount of alkaloid initially consumed. The peak areas of samples from predators that had consumed eggs were divided by the mean alkaloid peak areas of the three-egg samples in order to estimate how much of the initial prey alkaloid remained in the predator samples.

RESULTS

Isopropyleine from the historical, suitable prey *P. japonica* declined in the body of *H. axyridis* larval predators far more rapidly than that of the novel, less suitable alkaloid, adaline from *A. bipunctata* (Fig. 2). Isopropyleine reached <10% of its original quantity within 8 h and ≤1% within 32 h. By contrast, >10% of adaline still remained in the *H. axyridis* larval body after 32 h.

Both isopropyleine from *P. japonica* and adaline from *A. bipunctata* were recorded in haemolymph samples (Fig. 3). Isopropyleine was detected in all haemolymph samples collected directly after consumption of *P. japonica* eggs but no adaline was detected in samples collected directly after *A. bipunctata* eggs had been eaten. No isopropyleine was detected in haemolymph samples collected more than 4 h after egg consumption. Adaline, by contrast, continued to be detected in some samples up to 32 h after egg consumption.

Frass collected after *P. japonica* egg consumption contained a mean of 7.1% of the estimated original amount of isopropyleine consumed (range 3.5–8.9%, N=4). Frass collected after *A. bipunctata* egg consumption contained a mean of 16.1% of the estimated original amount of adaline consumed (range 8.3–31.7%, N=4).

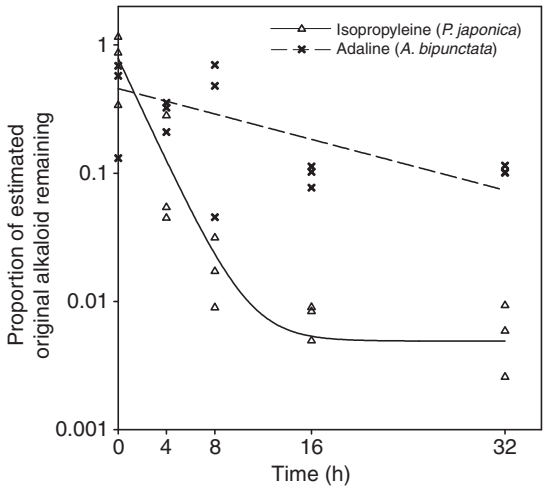

Fig. 2. Scattergram of the proportion of the two alkaloids, isopropyleine from *Propylea japonica*, and adaline from *Adalia bipunctata* remaining in fourth instar *Harmonia axyridis* larvae different time periods after egg consumption. Note the logarithmic *y*-axis. N=3 for each alkaloid and time period. Regression lines: isopropyleine, $y=0.0049+0.78e^{-0.47x}$, adjusted $R^2=0.75$, $F_{2,12}=21.77$, $P=0.0001$; adaline $y=0.46e^{-0.057x}$, adjusted $R^2=0.30$, $F_{1,13}=6.98$, $P=0.02$.

DISCUSSION

Harmonia axyridis performs well on a diet of the isopropyleine-bearing *P. japonica* with which it historically co-occurred and commonly interacted with. Performance is poorer with *A. bipunctata*, possessing novel alkaloids, most notably adaline, not known from any species in the native range of *H. axyridis*. The difference in the tolerance of *H. axyridis* for isopropyleine and adaline is reflected in their ADME profiles within the predator. Isopropyleine declines more rapidly than adaline in the larval body; it also enters and declines in the haemolymph more rapidly, and occurs less in frass.

The more rapid loss of isopropyleine from the body suggests that the predator has a specialised metabolism that renders the alkaloid less harmful through chemical modification of the alkaloid's structure, either by breaking it down or conjugating it. By contrast, *H. axyridis* does not apparently possess enzymes to chemically neutralise adaline, because this persists in its original form in the body for longer. Adaline is most likely ultimately changed by slower, non-specific processes. Changes in isopropyleine do not appear to occur in the gut as this alkaloid, like adaline, enters the predator's haemolymph. This occurs near-instantaneously after *P. japonica* egg predation, and this rapidity suggests active isopropyleine transport from the gut. Slower entry of adaline into the haemolymph is, by contrast, more probably a passive, non-specific process. The rapid disappearance of isopropyleine from haemolymph suggests that it is chemically altered there or is transported to another body compartment where such changes occur. Adaline persists in the haemolymph for longer, again indicating an absence of specific processes for handling this alkaloid. The levels of both alkaloids in frass are relatively low and, if anything, lower in the case of isopropyleine. Excretion of alkaloid unchanged from the gut does not, therefore, appear to be of great importance in toxicity avoidance. The lower proportion of isopropyleine in frass could be a correlate of its active transport into the haemolymph. It is noteworthy that *H. axyridis* transports isopropyleine into the haemolymph rather than detoxification occurring in the gut, as occurs in many insect herbivores. Target site insensitivity could exist alongside the metabolic mechanism, given the likely exposure of organs in the haemolymph to transported alkaloids.

Hautier et al., who used GC-MS of consumed adaline as a means to detect *H. axyridis* predation of *A. bipunctata*, suggested that its persistence might be because the ladybird sequesters the alkaloid for its own chemical defence (Hautier et al., 2008). Such a defence would act against a diversity of natural enemies, including generalist predators such as birds, ants and spiders as well as intraguild predators (cf. Majerus, 1994; Ceryngier and Hodek, 1996). Hautier et al., however, lacked similar data for other prey species with which

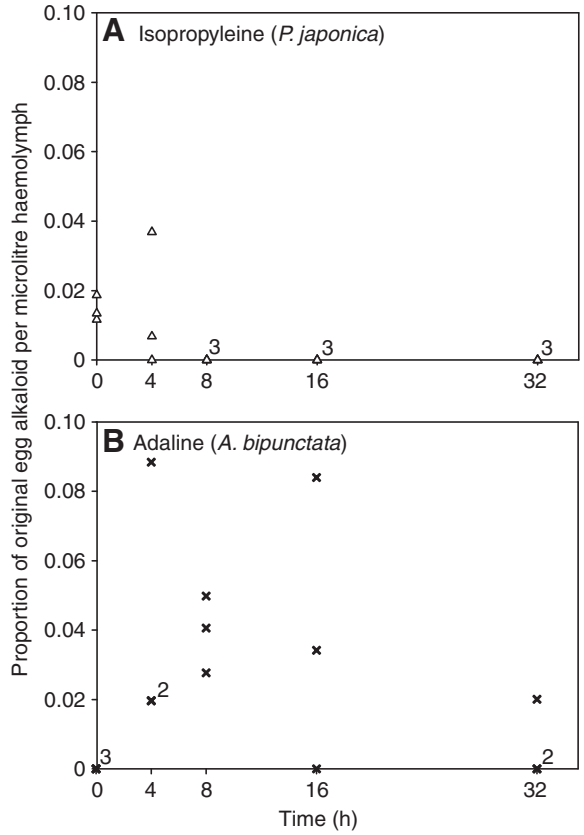

Fig. 3. Scattergram of the proportion of (A) isopropyleine (B) adaline remaining in 1 µl of *Harmonia axyridis* larval haemolymph different time periods after egg consumption. *N*=3 for each alkaloid and time period; a number by a point indicates that the point comprises that number of data points superimposed on each other.

to compare the persistence of adaline (Hautier et al., 2008). The results described in our study indicate that sequestration of prey alkaloids does not occur in *H. axyridis*. More consistent with the data is that *H. axyridis* lacks the biochemical pathways for altering adaline to render it harmless. This is indicated by the more rapid metabolism of isopropyleine, to which *H. axyridis* is adapted, the known toxicity of *A. bipunctata* eggs to *H. axyridis* and the lack of a coevolutionary history between *H. axyridis* and *A. bipunctata*. Long adaline persistence in an unmodified form is therefore linked to its toxicity to *H. axyridis*. It cannot be completely ruled out that prey alkaloids tolerated by *H. axyridis* are stored for defence in a less harmful form, as is known to occur in some alkaloid-sequestering herbivores (Lindigkeit et al., 1997; Narberhaus et al., 2004); this is particularly so as prey alkaloids do enter the haemolymph where the defensive alkaloids of ladybirds are stored. Similarly, it remains possible that there is a transient benefit as alkaloid is transported through the haemolymph. An interesting opportunity resulting from the occurrence of prey alkaloids in haemolymph is that GC-MS of defensive secretions could be used to screen non-destructively for the alkaloids of ladybird prey in studies of IGP in the field: the diversity of previous methodological approaches, including GC-MS based ones, have all involved killing the predator (see Weber and Lundgren, 2009).

Our findings emphasise that the poor performance of *H. axyridis* on prey encountered outside its native range and possessing novel alkaloids has a physiological basis. This is centred on a relatively high degree of metabolic specialisation. Generalist herbivores appear to utilise metabolic enzymes that are structurally and functionally more flexible than those of specialists (Li et al., 2004), and although studies of generalist herbivory in the context of the enemy release hypothesis vary in their findings (e.g. Parker and Hay, 2005; Branson and Sword, 2009), at least one study indicates that native generalists can exhibit some metabolic tolerance of exotic, novel plant allelochemicals (Castells and Berenbaum, 2008). The higher specificity in our study of a predator compared with studies of herbivorous generalists is noteworthy. Predators would be expected to have more in common with generalist than specialist herbivores, by virtue of the number of different types of prey they consume. This is certainly true of *H. axyridis*, which possesses one of the broadest ranges of prey aphid species, in addition to consuming ladybirds and possibly other types of invertebrates (Iablokoff-Khnzorian, 1982; Koch and Galvan, 2008). Its prey possess diverse forms of defensive chemistry. Furthermore, in many cases, prey appear to be consumed in sufficient quantities to suggest that *H. axyridis* does not simply avoid the physiological burden imposed by individual toxins by consuming a wide diversity of chemically prey in individually very small amounts (cf. Skelhorn and Rowe, 2007).

This poses an interesting problem: if prey allelochemical metabolism is specific how can a generalist predator apparently consume so many different types of prey? High metabolic specificity might be expected to lead to strong genetic trade-offs in the tolerance of different prey defensive chemicals, leading to a higher degree of prey specialisation. The limited evidence relating to prey chemistry-based trade-offs in invertebrate predators is mixed (Albuquerque et al., 1997; Sadeghi and Gilbert, 1999; Pekár et al., 2008), and in aphidophagous ladybirds such trade-offs have been suggested to be of limited importance compared with trade-offs related to prey capture, notably body size (Sloggett, 2008a, Sloggett, 2008b). Clearly additional work is required to link metabolic tolerance and the role of trade-offs in predators. *Harmonia axyridis* makes an ideal model organism with which to gain further insights into the relationship between the two, due to the abundant work already in existence on its feeding ecology and diet. At least with respect to the alkaloids of its ladybird prey, *H. axyridis* clearly exhibits a specialised alkaloid metabolism, which is evidently not shared by many other ladybirds. Many studies have attempted to explain why *H. axyridis* is uniquely well adapted as a competitor and predator of other ladybird species. Our study goes some way to explaining this, while at the same time illustrating that even such a notorious predator as this faces physiological limits imposed by its evolutionary history.

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