Intraspecific and Interspecific Comparison of Toxicity of Ladybirds (Coleoptera: Coccinellidae) With Contrasting Colouration

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Research Article

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Abstract

Ladybirds (Coccinellidae) use toxic compounds, mostly alkaloids in their haemolymph, for defence against predators and other enemies. The toxicity of ladybirds to predators cannot be directly assessed because predators show avoidance reactions without ingesting the beetles. The alkaloid of ladybird *Harmonia axyridis* showed wide range toxicity to diverse non-target organisms. Thus, we used a quick, inexpensive and easy-to-perform method using bioassays on water flea *Daphnia magna* for comparative quantification of the toxicity (LD_{50}) of whole body extracts from several species of ladybirds that differ in their warning colouration. Alien invasive aposematic polymorphic ladybird *H. axyridis* was more toxic than all the other species examined: aposematic *Adalia bipunctata* > cryptic *Cynegetis impunctata* > aposematic *Coccinella septempunctata* > slightly aposematic *Calvia quatuordecimguttata*. Three months old adults of *H. axyridis* were 3.8 times more toxic than two weeks and one month old adults. The two most common colour morphs (non-melanic *novemdecimsignata* and melanic *spectabilis*) did not differ in their toxicity.

Introduction

Chemical compounds play various roles in the ecology of ladybirds (Coleoptera: Coccinellidae), involving searching and consuming food, recognizing mates and competitors, aggregating and protection against natural enemies [1]. Adult ladybirds produce a droplet of malodourous and distasteful haemolymph from the tibio-femoral joints in a process known as reflex bleeding, which functions as both mechanical (sticky) and chemical protection [2]. Protective chemical substances include mainly alkaloids (bitter taste, potentially toxic [3] [4] and pyrazines (smelly but non-toxic, [5]).

Many predators find the defensive chemicals of ladybirds distasteful or toxic [6] [7]. The varying degree of response and toxic effects on the predators are due to the differences in the alkaloid identity specific for ladybird genera [8] and in their age [9]. *Coccinella septempunctata* caused toxic effects and resulted in severe liver damage in the nestlings of blue tits *Cyanistes caeruleus* [6]. Other studies of bird-ladybird interactions showed strong repellence effect precluding feeding the beetles and thus manifestation of their real toxicity (by tits *Parus major*, domestic chicks) or no effect after ingestion (in sparrow *Passer montanus* [10]). Ladybird reflex blood has been also found distasteful to ants [7, 11, 12]. Ladybirds are sometimes preyed on by spiders, particularly web-building spiders [13]. Nonetheless, even if trapped in the spider web, ladybirds may not be consumed due to the presence of their defensive chemicals [14].

There are also findings about toxicity of ladybirds to non-predatory, non-target organisms, including pathogenic microorganisms. The research group of Andreas Vilcinskas found that toxicity of the alkaloid harmonine of *Harmonia axyridis* displayed antimicrobial activity against *Mycobacterium tuberculosis* and *Plasmodium falciparum* [15]. Haemolymph of *H. axyridis* suppressed multiplication of *Escherichia coli* [16]. Germination of seeds and growth of root of seedlings of *Sinapis alba* were inhibited by extracts from *H. axyridis* [9].

The first use of a standard toxicity assay using cladoceran *Daphnia magna* [17] for quantification of ladybird chemical defence [18] revealed that *H. axyridis* was more toxic than *C. septempunctata* and *Adalia bipunctata*. Usefulness of this test was later confirmed by Arenas et al. [19], who report toxicity of several native British species of ladybirds to *Daphnia pulex*, ordered approximately according to the strength of their warning colouration: *Halyzia sedecimguttata* > *A. bipunctata* > *Exochomus quadripustulatus* > *Propylea quatuordecimpunctata* > *Aphidecta obliterata* > Control. The water flea *D. magna* is an important crustacean species inhabiting aquatic ecosystem having Holarctic distribution. Different aspects of its ecology, life history, genetics and reactions to changes in environment and to toxic effects of chemicals are extensively studied [20]. *Daphnia magna* is used in aquatic toxicology [21] primarily for its ease of culture, its high sensitivity to toxins and its clonal method of reproduction [22]. The usual temperature at which toxicity tests for water fleas are performed is about 20°C, because it does not vary much from room temperature [22].

Ladybirds are famous for their colour patterns, which are mostly aposematic, warning about their unpalatability or distastefulness [23]. Insectivorous birds exhibit avoiding behaviour against ladybirds species with diverse colour pattern [24], including immature stages [10, 25]. Many ladybird species include several distinct colour forms. Birds moderately distinguished between diverse colours and patterns of polymorphic *H. axyridis* (spotted individuals better protected than unspotted [26], melanic *spectabilis* more often attacked than non-melanic *novemdecimsignata* [27]. Arenas et al. [19] did not find difference in toxicity of two different forms of *A. bipunctata*. Sakaki and Nedvěd [9] found only minor differences in phytotoxicity among 13 colour morphs of *H. axyridis*.

Toxicity of ladybirds usually increases with the age. Extracts from one week old adults of *H. axyridis* caused suppression of root growth of seedlings of white mustard to one third, extracts from three months old ladybirds decreased the root length to one tenth [9]. The killing efficiency of the haemolymph of *H. axyridis* against *Escherichia coli* increased from larval stages through the prepupal stage and during the entire period of adult life [16].

Harmonia axyridis is an ideal model for studying warning signals and toxicity of ladybirds. It is a large and highly toxic ladybird [18] found in many colour forms [28] induced genetically [29] or environmentally [30]. The alkaloids of *H. axyridis*, harmonine, like those of the other ladybirds, provide protection against a number of invertebrate and vertebrate predators suggesting its strong chemical defences [31]. It is a very successful alien invasive species with many superior properties, including chemical defence [32].

Because it is impossible to measure the direct acute toxicity of ladybirds to their predators, as they show various avoidance reactions without eating the beetles (see above), and because of the broad spectrum of organisms in which the defensive compounds of ladybirds appeared toxic, we decided to employ a simple standard test of the toxicity of whole body extracts from several species and morphs of ladybirds with different warning colouration for the water flea, *D. magna*. We expected that i) young ladybirds would be less toxic than older ones; ii) more aposematic (red and black) species would be more toxic than less aposematic and cryptic ones; iii) colour morphs of a polymorphic ladybird species would not differ in effects of their defensive chemicals.

Table 1

Lethal effects of extracts from several species and morphs of ladybirds on water flea *Daphnia magna*. Haxy: *Harmonia axyridis*, spec: f. *spectabilis*, others f. *novemdecimsignata*; 2w: two weeks old adults, 4w: 4 weeks, 3m: 3 months, A2P: *Adalia bipunctata*, CIMP: *Cynegetis impunctata*, C7P: *Coccinella septempunctata*, C14G: *Calvia quatuordecimguttata*. BM: average fresh body mass (mg); Control: survival percentage of *D. magna* in water without extract after 24 hour exposure related to specific experimental group; R: regression coefficient of the logistic equation $Y=\exp(b*(x-C_{50}))/(1+\exp(b*(x-C_{50})))$ describing the effect of extract dose on survival; b: regression parameter describing the slope of decrease of survival ± SE; C_{50}: concentrations (mg/ml) killing 50% of *D. magna* ±SE.

Group	BM	Control	R	b±SE	C_{50}±SE	
Haxy 3m	36.8	96	0.697	−23.1±6.7	0.0595±0.0086	A
Haxy spec	35.7	96	0.766	−25.3±6.3	0.0742±0.0082	A
Haxy 2w	34.6	80	0.821	−29.0±12.9	0.221±0.014	B
Haxy 4w	34.5	80	0.941	−99.5±43.9	0.233±0.008	B
A2P	9.7	86	0.888	−25.6±5.9	0.270±0.010	C
CIMP	5.7	84	0.868	−8.7±2.3	0.464±0.037	D
C7P	43.9	81	0.815	−3.92±1.06	0.863±0.079	E
C14G	19.1	90	0.868	−2.45±0.45	1.232±0.098	F

Letters indicate significant differences in C_{50} according to GLM Analysis of covariance.

Results

Effect of all eight groups of ladybirds (species, colour morphs, age cohorts) on survival of water flea was highly significant in the GLM model ($Wald_7=122$, $p<10^{-6}$). We found no difference between 2 weeks and 4 weeks old adults of *H. axyridis* but the toxicity of 3 months old adults was 3.8 times higher (Tab.1, Fig. 1). We found non-significant difference between melanic and non-melanic morphs of *H. axyridis*. All other species examined were less toxic than *H. axyridis*. *A. bipunctata* was about 1.2× and *C. septempunctata* about 3.8× less toxic to *D. magna* than young *H. axyridis*. The cryptic *Cynegetis impunctata* was moderately toxic (2× less than young *H. axyridis*) and the slightly aposematic *Calvia quatuordecimguttata* were least toxic (5.4× less than young *H. axyridis*, Fig. 2).

Discussion

Our study confirms previous findings [18, 19] that whole body extracts containing the defensive chemicals of ladybirds such as alkaloids may be lethal for the water flea *D. magna* and thus this standard toxicological assay can be used for comparative quantification of ladybird toxicity.

Toxicity and invasive success

We found that *H. axyridis* was more toxic to *D. magna* than the other ladybirds. This finding is similar to our previous results [18] where the lethal concentration (C_{50}=0.06 mg/ml) was many times lower than that of other two conspicuous aposematic ladybirds *A. bipunctata* (0.6 mg/ml) and *C. septempunctata* (4 mg/ml). Our present results confirms the order of species in toxicity levels measured in the previous study, but not the magnitude of the interspecific differences. Age can be responsible for the differences (see below). It is believed that the high toxicity is one of the factors that can help the invasive alien species *H. axyridis* to be so successful in establishing in new areas [32].

Several studies have been conducted to characterize the ecological aspects, properties and biological functions of harmonine, the alkaloid of *H. axyridis*. Ants *Myrmica rubra* showed deterrence to harmonine at concentrations of 10^{-4} M revealing the protective function of harmonine against invertebrate predators [33]. In our related study [12] we found only small differences between six species of ladybirds in repellence to ant *Lasius niger*. A recent study explored the presence of parasitic microsporidia in the haemolymph of *H. axyridis* [34]. Although detrimental to other coccinellid species, these microsporidia do not affect *H. axyridis* [35] and thus harmonine protects *H. axyridis* from self-infection [36].

Aposematism and toxicity: interspecific comparison

We consider the brown-and-white *C. quatuordecimguttata* as moderately aposematic, while it was the least toxic ladybird among our species. Another study using bioassays on *D. pulex* with ladybird toxins extracts [19] included somewhat similar (orange-and-white) *H. sedecimguttata* which was the most toxic among their ladybird species analysed. Thus, toxicity of ladybirds with such type of pattern is quite unpredictable.

The cryptic ladybird *C. impunctata* that could be assumed less chemically defended against predators than the aposematically coloured species was in fact moderately toxic to water fleas in our study. In the study by Arenas et al. [19], the non-aposematic *Aphidecta obliterata* was the least toxic among the species used. Thus, cryptic species seem to show little to moderate toxicity.

In our study, aposematic *A. bipunctata* appeared to be more toxic than other species except *H. axyridis*, and it was also the second most toxic species in the study by Arenas et al. [19]. They considered both melanic and typical morphs of this species very aposematic (according to contrast and colour saturation). In our study, *A. bipunctata* was more toxic than *C. septempunctata*, while Arenas et al. [19] did not study the latter species. Repellence by *A. bipunctata* for ants (expressed as concentration repelling half of individuals, C_{50}) was also higher [12]. These findings are in contrast to the toxicity of the two species for the blue tit *C. caeruleus* where only *C. septempunctata* killed the nestlings [6]. It indicates lower efficacy of chemical protection in *A. bipunctata* against vertebrate predators, despite the presence of a higher concentration of alkaloids than in *C. septempunctata* [37]. Thus, the standard toxicity test using *Daphnia* species need not show accurate differences between toxicity of ladybird species against bird predators.

The hypothesized positive relationship between the aposematism and toxicity in ladybirds (called signal honesty [19]) was not supported in our study. More species of ladybirds is needed in future studies to support or falsify possible hypotheses about the role of colouration, body size, food specificity and habitat preference on their toxicity level.

The invasive *H. axyridis*, apparently as much aposematic as *A. bipunctata* and *C. septempunctata*, was the most toxic in our study. The order of repellence to ants was different: *A. bipunctata* > *H. axyridis* > *C. septempunctata* [12]. It is notable that dead individuals of *C. septempunctata* were less scavenged by invertebrates (more repellent) than otherwise highly toxic *H. axyridis* [38]. Antimicrobial activity of haemolymph of *H. axyridis* against *Escherichia coli* was 4 times greater than that of *C. septempunctata* [39]. We conclude that toxicity of individual species of ladybirds to diverse predators differs from repellence and from antimicrobial activity, the former probably being caused by alkaloids, the second by pyrazines, the third by alkaloids and peptides.

Aposematism and toxicity: intraspecific comparison

We observed no difference between melanic (*spectabilis*) and non-melanic (*nevemdecimsignata*) morphs of *H. axyridis*, thus showing that colour morphs may not differ in effects of their defensive chemicals. It is in accordance with the study by Arenas et al. [19] in which the extracts from melanic and non-melanic forms of *A. bipunctata* showed no differences in toxic effects on *D. pulex*. In other study [9], we compared phytotoxicity of 13 colour morphs of the polymorphic *H. axyridis* without consistent differences. Colour morphs of *H. axyridis* collected in the field in Czechia did not differ significantly in the parasitization rate by fungus *Hesperomyces virescens* and infection rate by *Spiroplasma* [40], while in wider center-European comparison, the melanic colour forms *conspicua* and *spectabilis* were less often parasitized than non-melanic form *novemdecimsignata* [41].

Fischer et al. [42] reported non-melanic *H. axyridis* with pale-orange colour possessing a higher content of harmonine than melanic individuals. Nevertheless, Sloggett [43] observed almost equal repellence to invertebrates by melanic and non-melanic *H. axyridis* mixed to food. Fischer et al. [44] observed lower production of methoxypyrazine by red individuals than other colour forms. The alkaloid level was negatively correlated with the extent of melanic pattern on the elytra of the non-melanic *H. axyridis* [45]. Revealing the importance of methoxypyrazines as warning odours for repellence and toxicity, Fischer et al. [42, 44] accomplished no correlation between methoxypyrazine emission and harmonine content in *H. axyridis*. This confirms the above mentioned interspecific difference between repellence and toxicity.

Age and toxicity

Although we did not find difference in toxicity between ages 2 weeks and 4 weeks, the toxicity of much older adults (3 months) was 3.8 times higher. Similarly, phytotoxicity assay [9] showed much stronger effect caused by extracts from 3 months old adults than from 1 week old ones. The carotenoid pigment uses to accumulate throughout the life of a ladybird resulting in the darkening of elytra [23, 46]. However, there was no relationship between alkaloid content and either elytra redness or carotenoid pigment concentration in either sex of field collected *H. axyridis* [45]. Younger orange individuals had higher number of body zones with thalli of the parasitic fungus *H. virescens* than red individuals [40], but older red individuals were not protected against *H. virescens* [47]. We suggest that some inconsistency between various studies regarding the relationship between age and toxicity can be ascribed to differences between laboratory reared and field collected ladybirds, although Arenas et al. [19] report indistinguishable toxicity of bought and wild-caught individuals of *A. bipunctata*.

Materials And Methods

Experimental animals. Water extracts from individuals of the following species, colour forms and age cohorts of ladybirds were available in necessary large quantities for the experiment:

1. Haxy 2w: *Harmonia axyridis* Pallas f. *novemdecimsignata* (otherwise often called *succinea*), two weeks old adults from laboratory stock, fed with aphids *Acythosiphom pisum*;
2. Haxy 4w: *Harmonia axyridis* f. *novemdecimsignata*, four weeks old adults from laboratory;
3. Haxy 3m: *Harmonia axyridis* f. *novemdecimsignata*, three months old adults from laboratory;
4. Haxy spec: *Harmonia axyridis* f. *spectabilis*, three months old adults from laboratory;
5. A2P: *Adalia bipunctata* (Linnaeus) f. *typica*, one month old adults from laboratory;
6. C14G: *Calvia quatuordecimguttata* (Linnaeus), overwintering adults from native population, frozen at –20°C;
7. C7P: *Coccinella septempunctata* Linnaeus, overwintering adults from native population, frozen at –20°C;
8. CIMP: *Cynegetis impunctata* (Linnaeus), 4th instar larvae from native population.

Native populations originated nearby České Budějovice, Czechia.

Adult females of water flea *Daphnia magna* were collected from a local pond in České Budějovice (Czechia, 49°00'N, 14°26'E) during June 2019 and were maintained in the laboratory at 20°C, 16h L:8h D photoperiod. We added tap water that was allowed to reach the gas equilibrium, i.e. loose traces of chlorine and dissolve oxygen. Only non-pregnant females with high swimming activity were used for the assay.

Extraction. Body mass of each beetle and 4th instar larva was measured on balances with 0.1mg precision. Each individual was homogenized in 500μl of water by crushing them with a polypropylene piston and mixing them in an Eppendorf tube. After vortexing, the mixture was centrifuged at 20 000 RPM for 2 minutes, and supernatant separated. The pellet was extracted once more with the same volume of water. The merged two supernatants formed the unit experimental solution.

Toxicity assay. We used 50ml plastic cups containing 20ml of water and 10 water fleas. To evaluate the toxicity of ladybird extracts, diverse volumes of the unit experimental solution was added to the cup to make a binary log series of concentrations. Cups containing only 20ml pure water with 10 water fleas were used as control. Each concentration (volume) was replicated five times. The cups were kept at 20°C, 16h L:8h D and high air humidity to reduce evaporation of water from the cups. The number of immobilized water fleas was recorded after 24h exposure. Water fleas unable to swim for 15 seconds after gentle stirring were considered immobilized/dead [21].

These methods were carried out in accordance with relevant guidelines and regulations provided by Organisation for Economic Co-operation and Development. The study was carried out in compliance with the ARRIVE guidelines 1–4, 6–10.

Statistical analysis. We started with the logistic regression y=exp(a+b*x)/(1+exp(a+b*x)) using Statistica 13 software [40]. The independent variable was x=BM/(BM+1000)*V/20, i.e. fresh body mass (BM) of ladybird in mg divided by BM+1000 (mass of water added during extraction plus the mass of ladybird), multiplied by the volume (V) of extract used in a replication in microliters divided by 20 (added to 20 ml of tap water). Either survival (1) or mortality (0) was coded as the response variable (y) with counts of water flea individuals of respective fate to calculate the response of water fleas to the toxins of ladybirds. The concentration lethal to 50% of individuals (C_{50}) can be calculated as the ratio of the two equation parameters $C_{50}=-a/b$.

Then we used a modified function that estimates directly the C_{50} concentration (mg/ml) in the following form: $Y=\exp(b*(x-C_{50}))/(1+\exp(b*(x-C_{50})))$ in the Nonlinear estimation tool of this software package, because it provides calculation of standard errors (SE) of the parameters (see Tab. 1). Y states here for the survival rate Se in a group of 10 water fleas corrected by the survival rate of control Sc of the particular ladybird group (exposed to water), i.e. simplified Abbott's [49] formula: S=Se/Sc.

After Levene's test for normality, GLM tool Analysis of covariance with normal distribution and log link function provided comparisons (Wald statistics with 1 degree of freedom) of toxicity between the experimental groups. Effect of concentration was always highly significant ($p<10^{-6}$), and the effect of groups was then transferred to letters marking their differences (Tab. 1).

Declarations

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Author contributions

ON invented and designed the experiments, statistically analysed the results and finished the manuscript, MA performed the experiments and wrote draft manuscript.

Competing interests

The author(s) declare no competing interests.

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Figures

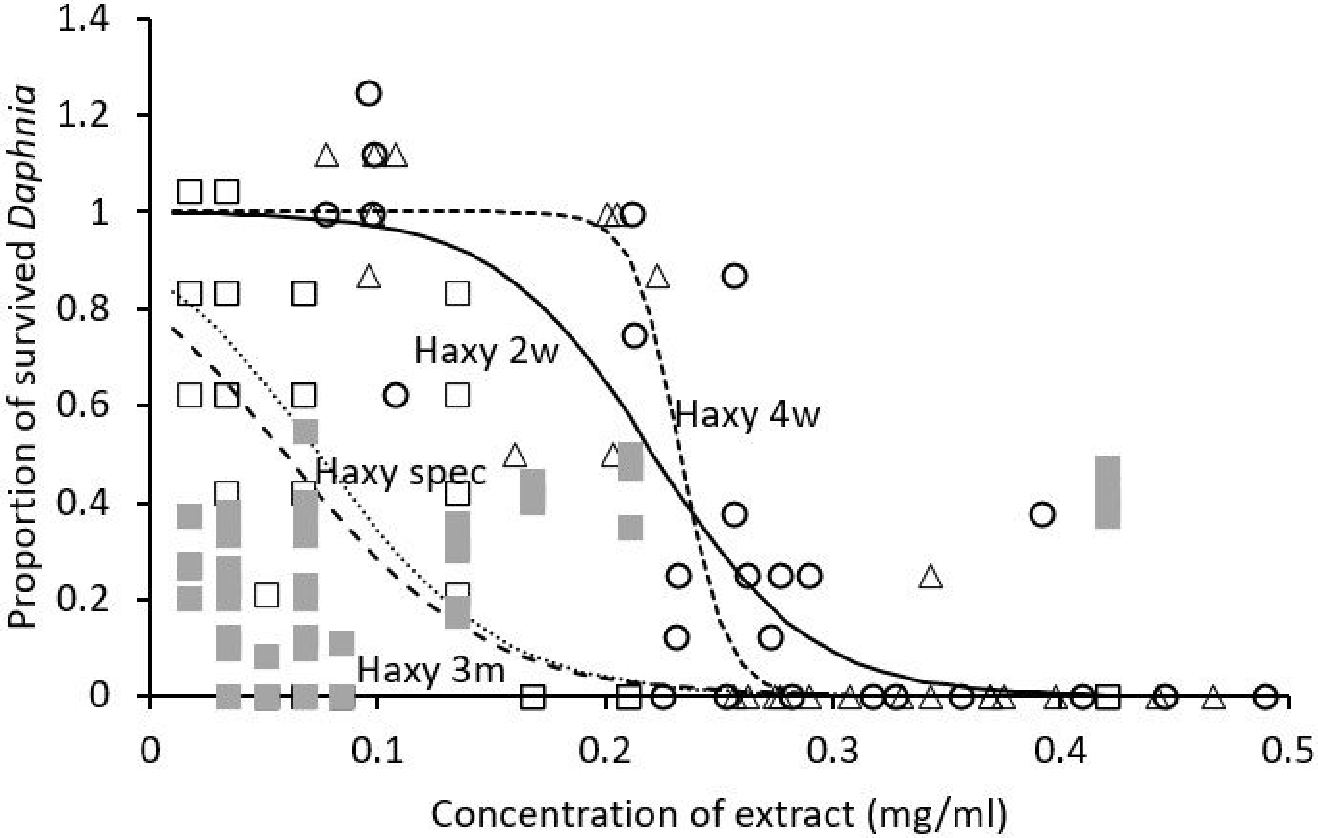

Figure 1

Effect of concentration of extract from ladybird *Harmonia axyridis* on survival of water flea *Daphnia magna* after 24 hours. Open squares and dashed line: Haxy3m f. *novemdecimsignata*, three months old adults; Grey squares and dotted line: Haxy spec f. *spectabilis*, three months old adults; Open circles and solid line: Haxy 2w f. *novemdecimsignata*, two weeks old adults; Open triangles and dashed line: Haxy 4w f. *novemdecimsignata*, four weeks old adults.

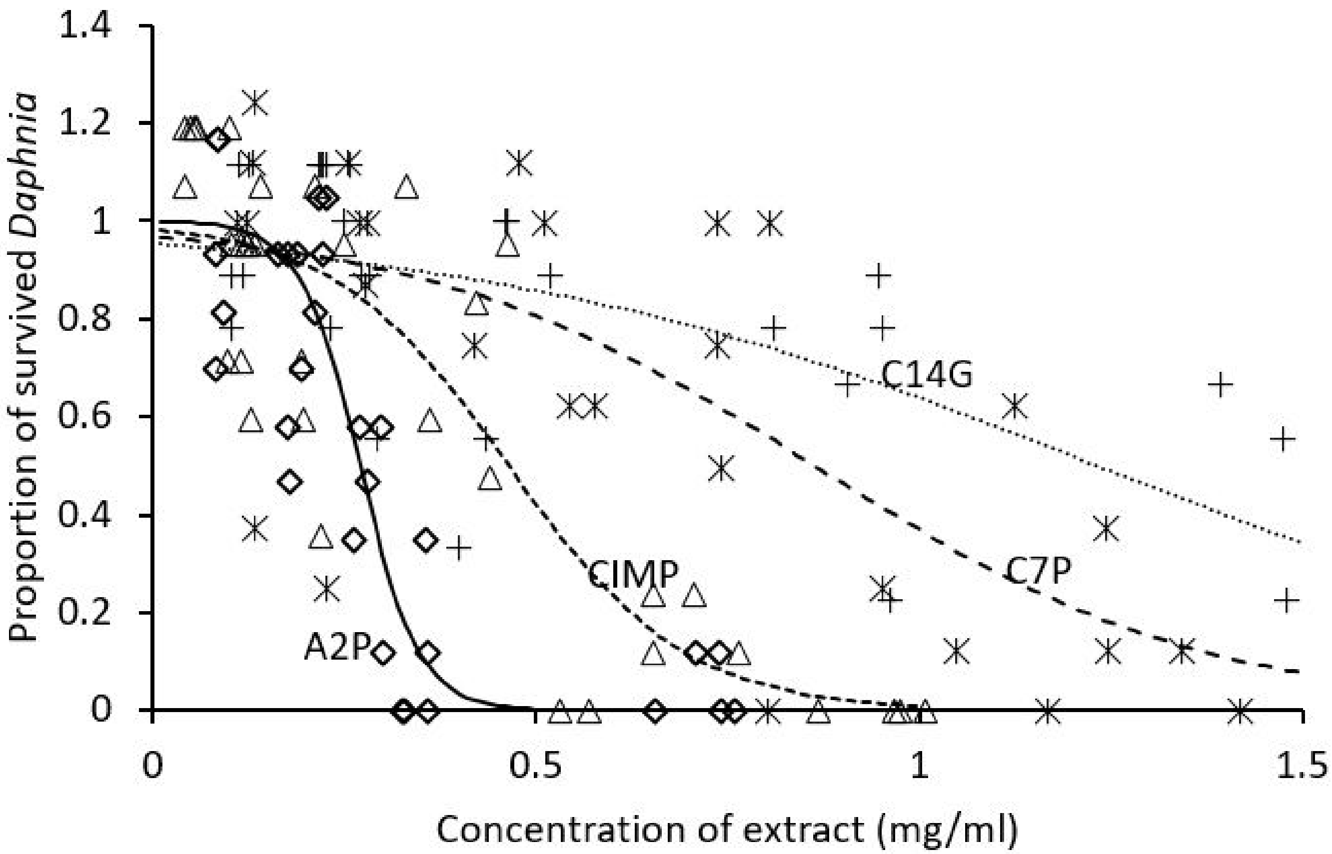

Figure 2

Effect of concentration of extract from ladybirds on survival of the water flea *Daphnia magna* after 24 hours. Diamonds and solid line: A2P *Adalia bipunctata*; Triangles and short dashed line: CIMP *Cynegetis impunctata* larvae; Stars and long dashed line: C7P *Coccinella septempunctata*; Crosses and dotted line: C14G *Calvia quatuordecimguttata*.