

Relative unpalatability of leaf beetles with either biosynthesized or sequestered chemical defence

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Abstract. Defence of leaf beetles in the genus *Oreina* is chemically diverse. Some (e.g. *O. gloriosa*) rely on the secretion of small quantities of a concentrated mixture of cardenolides that are biosynthesized de novo and stored only in special glands. Others (e.g. *O. cacaliae*) lack cardenolides but sequester pyrrolizidine alkaloid N-oxides (PAs) and store them both in their body and in their glands. First, the impact of the defensive secretions on the palatability of the beetles to wild-caught red-winged blackbirds, *Agelaius phoeniceus*, was tested. The reactions of the birds to these two different types of chemicals were then compared to determine whether they grant equal protection to the beetles. *Oreina gloriosa*, with their secretion of cardenolides intact, were eaten in 55% of the trials. When the secretion was physically removed, 95% of the now undefended *O. gloriosa* were eaten. The mean handling time by the birds also decreased from 41 to 26 s. This shows that the small quantity of highly concentrated secretion produced on the surface of the beetle's body affords substantial protection against avian predators. *Oreina cacaliae*, with PAs in their secretion and in their body, were eaten in only 21% of the cases. When the secretion was removed, leaving PA only in the body, the number eaten rose to 36%. The time to peck and then reject the beetles with their secretions intact was less in *O. cacaliae* than in *O. gloriosa*. PAs therefore seem to provide the beetle with better protection from the birds than do cardenolides. The experiment raised the possibility that the birds may have the ability to reject beetles with PAs olfactorily.

Chemical defence of leaf beetles of the genus *Oreina* (Chrysomelidae, Coleoptera) is diverse (review in Pasteels 1993; Pasteels et al. 1992, in press). Some species (e.g. *O. gloriosa*) rely on small quantities (24–8 µg per beetle) of highly concentrated (0.27 M) cardenolides (Eggenberger & Rowell-Rahier 1993). These cardenolides are biosynthesized de novo and stored exclusively in pronotal and elytral glands (Pasteels et al. 1992). When the beetles are disturbed, they liberate the contents of their glands and spread them out over the cuticle. In other species (e.g. *O. cacaliae*), the beetles sequester pyrrolizidine N-oxides (PAs) from asteraceous host-plants in both their body and in their glands (Pasteels et al. 1988; Ehmke et al. 1991; Rowell-Rahier et al. 1991). In *O. cacaliae* the total toxin content (102 µg per beetle) is higher than in *O. gloriosa* but the secretion is less concentrated (4.2 µg at 0.17 M) and the majority of the PAs are stored in the body (98 µg).

Sequestration of PAs and cardenolides occurs in several Lepidoptera, and is generally considered to be a primitive form of defence compared with the de novo synthesis (Brown et al. 1991). However, it has been suggested elsewhere that PA sequestration in *Oreina* spp. evolved subsequent to the de novo synthesis of cardenolides that typifies many Chrysolinina (review in Pasteels et al. 1992, in press).

Cardenolide-mediated protection of herbivorous insects is well known in many species, including the famous example of their sequestration from Asclepiadaceae by monarch butterflies, *Danaus plexippus* (Brower 1984; Brower et al. 1988; Malcolm 1991). However, the amount of cardenolides present in leaf beetles is less than 20% of that found in the average monarch.

The protective role of pyrrolizidine alkaloids is less well documented (Boppré 1986) even though numerous Lepidoptera as well as other insects are able to sequester PAs from their food plants and

store them in their body (Boppré 1990; Hartmann 1991). Brown (1984) has shown that the spider *Nephila clavipes* refuses Ithomine butterflies as prey when these contain PAs. Here again the quantity present in these Lepidoptera is much larger (up to 20% dry weight) than the amounts found in the leaf beetles. While pyrrolizidine alkaloids have been clearly shown to deter spiders (Brown 1984; Masters 1990), experiments testing their role as deterrents to lizards, birds and mice have been somewhat equivocal (Glendinning 1990, 1993; Glendinning & Brower 1990; Masters 1992).

The purpose of our experiment was to test the effect of the defensive secretion on the palatability of the beetles to birds. Specifically, we asked are the small quantities of the two kinds of toxins found in the secretions of the beetles sufficient for their defence? Second, we compared the palatability of beetles defended either by cardenolides or pyrrolizidines (biosynthesized or sequestered chemicals, respectively) to examine whether both types of beetles are equally protected, and thus perhaps to postulate an evolutionary advantage of the one type of defence relative to the other.

METHODS

Leaf Beetles

We selected two *Oreina* species for our study, each being characterized by only one of the chemical classes under scrutiny. We chose to compare these two species because they look very much alike, and are the same size (1 cm long) and colour (blue with green stripes). Thus the major variable being compared is that one has PAs and the other has cardenolides. The beetles were divided into four experimental groups: (1) *O. gloriosa* with the cardenolide secretion intact in the glands (C^+); (2) *O. gloriosa* without cardenolide secretion in its glands (C^-); (3) *O. cacaliae* with PAs in the body and in the glands (PA^{bs}); and (4) *O. cacaliae* without PA secretion in the gland, but with PAs in the body (PA^b). To remove the secretions, the beetles were stimulated (tapped gently on their pronotum and elytra with a pair of fine forceps) and then wiped with a piece of clean filter paper. Immediately prior to each feeding experiment the beetles were cooled briefly on dry ice to reduce their mobility.

Birds

Male red-winged blackbirds, *Agelaius phoeniceus* (Icteridae), captured from the wild in the southeastern U.S.A. were used as naive predators. These birds include substantial numbers of insects in their diet but they could never have encountered either of the *Oreina* species because they are native to Europe. Although the birds are probably familiar with small blue and green beetles and with insects containing cardenolides and PAs (e.g. Lepidoptera), we think it unlikely that they could have encountered beetles with these chemical and visual characteristics in eastern North America.

The birds were kept singly in experimental cages measuring $76 \times 76 \times 76$ cm, at 22°C with artificial daylight between 0800 and 2200 hours. They were visually isolated from each other, and from the experimenters by a one-way glass partition in the front of the cage. The birds were familiarized with and trained to feed from a rotating lazy susan feeder as described in Coppinger (1969). They were starved 3 h (0900–1200 hours) before the daily experiments and fed ad libitum with crickets, mealworms and dry poultry diet after each experimental feeding session. Water was always available. Each bird was tested no more than five times per day and on only 2 consecutive days. The number of trials per day and per bird (three to five) was dependent on the number of birds available for testing (i.e. trained to feed in the rotating lazy susan feeder). The experiment always stopped at 1600 hours to allow all the birds to feed for several hours before dark. Thus variation in hunger level of the different individual birds was kept minimal.

Some birds failed to meet the experimental criteria (see below) and were excluded from the experiment.

Bioassay

Each test consisted of successive presentations, in the rotating lazy susan cups, of one control mealworm and two beetles, each from a different experimental group. The order of the presentation was completely randomized in each test (with a random number generator). The interval between presentations of each individual insect was 2 min, and 20 min were allowed to elapse between successive tests on the same bird. The data analysed are based only on those birds that accepted and ate each mealworm in less than 2 s.

Table 1. Post hoc cell contributions for beetle group and behavioural category (standardized residual for each cell in chi-squared statistic from Fig. 1)

	Not tasted	Pecked	Partially eaten	Completely eaten
C ⁻	-2.73	-3.72	-2.86	6.38
C ⁺	-2.85	3.75	1.24	-1.56
PA ^{bs}	5.07	0.25	1.97	-4.86
PA ^b	3.26	-0.67	0.08	-1.82

C⁺: *O. gloriosa* with secretion intact; C⁻: *O. gloriosa* with secretion removed; PA^{bs}: *O. cacaliae* with secretion intact; PA^b: *O. cacaliae* with secretion removed.

The behaviour of the birds was scored and their time to manipulate the beetles ('manipulation time') measured. The behavioural categories scored were (1) not tasted, suggesting visual (or olfactory) rejection; (2) pecked and then dropped, suggesting gustatory rejection; (3) partially eaten, suggesting low palatability; and (4) completely eaten, suggesting high palatability. Manipulation time in each behavioural category was used to compare the relative palatability of the different groups of beetles. The experiment was conducted over 6 days with 11 birds.

Data Handling

For statistical analyses we used Statview 4.0 on a Macintosh computer. First, we performed a two-way analysis of variance with manipulation time (in s) as dependent variable and behavioural score and experimental set of birds as categories (independent variables): it showed a significant effect of 'score' ($P < 0.0001$) but not of 'bird' ($P = 0.96$) or of the interaction ($P = 0.62$). Thus the manipulation time differs between behavioural categories but not between 'birds' tested and so pooling of data is justified. Thereafter, the data obtained with the different sets of birds were pooled for each behavioural category and each group of beetles.

RESULTS

Oreina gloriosa with their glandular secretion of cardenolides intact were eaten completely by the birds in ca 55% of all cases ($N = 69$), eaten partially in ca 11%, pecked at in ca 29%, and not tasted at all in only ca 4%. However, when the secretion was removed prior to the experiment, 95% of the now chemically undefended *O. gloriosa* ($N = 58$)

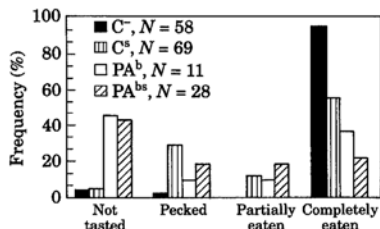


Figure 1. Frequencies (% of total observation per group) of bird responses for each defence group. C⁺: *O. gloriosa* with secretion intact; C⁻: *O. gloriosa* with secretion removed; PA^{bs}: *O. cacaliae* with secretion intact; PA^b: *O. cacaliae* with secretion removed.

were completely eaten (and none partially) and only 5% were either not tasted or pecked (Fig. 1).

Oreina cacaliae with PAs in their secretion and in their body were eaten completely in only 21% of all cases ($N = 28$). When the secretion was removed, leaving only the PA content of the body intact ($N = 11$), 36% were completely eaten. In about 40% of the presentations (12 from 28 and 5 from 11), the birds did not taste the beetles, either with or without secretion.

The difference in the birds' behaviour towards these defence groups is significant (Fig. 1; chi-squared test: $\chi^2 = 78.117$, $df = 9$, $P < 0.001$). Table 1 gives the post hoc cells. The largest contributions are 'not tasted/PA^{bs}' and 'eaten/C⁻'. Cardenolides seemed to cause gustatory rejection (pecking behaviour), whereas PAs were associated with visual (or olfactory) rejection (not tasted; Fig. 1). Also, as estimated by the proportion eaten completely, the palatability of a beetle attacked by a bird was always lower when it was protected by PAs present in both the body and the glands than by cardenolides (present only in the defensive glands), even in the absence of active secretion.

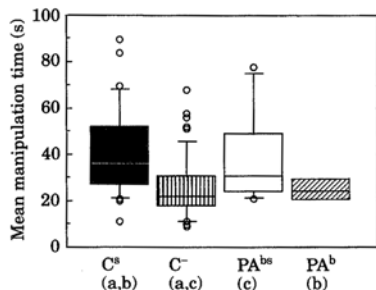


Figure 2. Mean manipulation time of beetles eaten by birds. Abbreviations as in Fig. 1. Data are given as box plots with five horizontal lines that display the 10th, 25th, 50th, 75th and 90th percentile of the variable time. Outliers are displayed separately. Groups marked with (a) on the horizontal axis are significantly different from each other ($P < 0.0001$) and those with (b) or (c) are not quite significantly different ($P < 0.06$).

In nature, prey necessitating a long manipulation time are probably not worth exploiting, even if palatable. Figure 2 shows the manipulation times of the completely eaten beetles belonging to the four groups. The results were submitted to a one-way ANOVA ($F_{3,99} = 7.138$, $P = 0.0002$) which indicates an effect of defence group on the manipulation time. For the completely eaten *O. gloriosa*, the manipulation time decreased significantly (Bonferroni test: $P < 0.0001$; Rice 1989) from a mean of 40.8 s ($SE = 3.0$, $N = 38$) to 25.9 s ($SE = 1.7$, $N = 55$) when the secretion was removed. The mean manipulation times of completely eaten *O. cacialae* with (38.8 s, $SE = 8.7$, $N = 6$) or without (25.0 s, $SE = 3.7$, $N = 4$) secretion are similar to those of *O. gloriosa* with or without secretion. The lack of significance when comparing PA^{bs} and PA^b may be caused by the smaller sample size for these treatments than for the C^- and C^s treatments.

These results demonstrate that the small quantity of highly concentrated secretion liberated on the surface of the beetle's body significantly increases the handling time of the beetles and therefore must contribute significantly to their protection against birds.

Shorter manipulation times of beetles that are pecked but not eaten probably also translate into higher survival of the beetles in nature. Figure 3 shows the manipulation times of pecked beetles

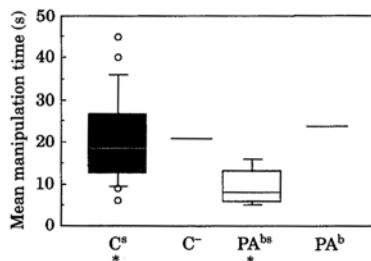


Figure 3. Mean manipulation time of beetles pecked by birds. Abbreviations as in Fig. 1. Data presentation is the same as in Fig. 2. * $P < 0.03$.

belonging to the four groups. Since in the groups C^- and PA^b , there is only one observation (respectively 24 and 21 s), the data were submitted to a Kruskal-Wallis non-parametric test: a nearly significant difference between the four groups was shown ($H = 7.393$, $df = 3$, $P = 0.06$). The approaching significance is due to the C^s and PA^{bs} data (Fig. 3). These two groups were run separately by ANOVA ($F_{1,23} = 5.158$, $P = 0.03$). The mean manipulation time of *O. cacialae* with secretion (PA^{bs}) was significantly and considerably shorter when they were pecked before rejection (9.4 s, $SE = 2.04$, $N = 5$) than in *O. gloriosa* with intact secretion (C^s) (20.2 s, $SE = 2.29$, $N = 20$). Thus, beetles that are pecked and released without being eaten might have a higher survival rate in nature if they contain PAs than if they contain cardenolides.

Our experiment was designed to avoid learning by the birds and change of behaviour with experience. For each of the two well-defended groups (C^s and PA^{bs}), we analysed the data for differences between the responses to their first presentation to a bird, to the second to fifth presentations and to the following ones. The behaviour of the bird did not change with successive encounters ($\chi^2 = 2.41$, $df = 6$, $N = 28$, $P = 0.88$ for the PA^{bs} beetles; $\chi^2 = 10.74$, $df = 6$, $N = 69$, $P = 0.10$ for the C^s beetles). Thus learning affecting the behavioural scoring can be excluded in our experiments.

We also checked for change in the mean manipulation time within each behavioural group: only in the PA^{bs} beetles is there a nearly significant correlation between decreasing pecking time and increasing presentation number ($r^2 = 0.72$,

$N=5$, $P=0.07$). The other experimental groups show no such relationship.

When the first encounter of the birds with the PA^{bs} and the C^s beetle groups are compared, there is a trend (Fisher exact test: $P=0.06$) towards more 'not tasted' observations in the PA^{bs} group (two out of four) than in the C^s group (none out of nine). In the light of this we cannot exclude the possibility that the beetles defended by PAs are rejected on the basis of olfaction.

DISCUSSION

In this study we compared the unpalatability of two closely related herbivore species, one that sequesters host-plant pyrrolizidines for use in its own defence and the other that produces defensive cardenolide *de novo*. The two chrysomelid species under scrutiny often occur sympatrically, their phenology is rather similar, they are aposematic and polymorphic in coloration (metallic blue and green), and they form locally abundant (patchy) populations. Additionally they are not distinguishable by the human eye and they are phylogenetically close (mt-DNA; T. Hsiao, personal communication). It is thus reasonable to assume that visually oriented predators such as birds will initially exert similar predation pressure on both species. Our laboratory experiments with an avian predator strongly suggest that plant-derived pyrrolizidine defence renders the beetles more unpalatable than the manufactured cardenolide defence.

If the PAs offer better protection against birds than cardenolides, the postulated switch in defence from biosynthesized cardenolides to sequestered PAs is easy to understand. However, one major drawback of sequestration is the unreliability of the source of toxins caused by well-known variation in plant content (Bowers 1992). Earlier, we have shown that *O. cacialae* buffers such variation by storing PA toxins in the body that can be mobilized to refill the glands after their depletion (Pasteels et al. 1992). The successful sequestration of PAs offers additional advantages over the biosynthesis of cardenolides: it gives better protection not only when the glands are full, but also when they are empty because of the toxin content of the body.

Our study demonstrates unambiguously the crucial role of the defensive secretion in rendering

the beetles unpalatable to red-winged blackbirds. Indeed, once their secretion was removed, *O. gloriosa* beetles became much more palatable. The localization of manufactured toxins in exocrine glands might be seen as an 'economic' advantage. Assuming that the biosynthesis of the toxins is costly, they should be used in the most parsimonious way, for example by releasing them in small quantities on the surface of the body where they come in direct contact with the aggressor only after stimulation by the attacking predator. We have shown here that these relatively small quantities released from the glands are effective. In comparison, lepidopterans that sequester PAs throughout their entire bodies would seem to be much less efficient than the beetles.

In addition, the release of toxins in the secretion plays an important role in reducing the value of the prey for the predator since it modifies handling time in two ways: (1) by increasing the time taken to eat the prey, and (2) for the PAs, by speeding up the taste rejection time.

PAs and cardenolides both have delayed toxic effects and we cannot exclude that there could be a learning asymmetry between the two sorts of toxins tested. Since our primary purpose was not to quantify learning, the rapid succession of trials and randomized presentation were used to avoid learned avoidance. However, for the pecking time of the PA^{bs} beetles, there is a correlation with the number of previous encounters.

One question remains completely open: how do the birds distinguish *O. cacialae*, with or without their secretion removed? Two possibilities are (1) they use some fine visual cue, or (2) they use olfactory discrimination. In our experiment it appears that the red-wing blackbirds rejected on sight more beetles that contain PAs than those that contain cardenolides, although there was no learning effect. To our knowledge the degree of olfactory discrimination by song birds is unclear. We cannot exclude the possibility that the modus operandi of PAs as vertebrate deterrents is that they may be sensed olfactorily. As such, following the classification of Brower (1984), they could operate either as noxious smelling class I defensive chemicals, or as class II defensive chemicals which operate as conditioned stimuli and warn the birds that the beetles contain other noxious compounds. Mason & Silver's (1983) discovery of odour aversion in starlings is of considerable interest in this context.

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