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Evolution

# Biparental defensive endowment of eggs with acquired plant alkaloid in the moth *Utetheisa ornatrix**

(pyrrolizidine alkaloid/defense/parental investment/nuptial gift/sexual selection)

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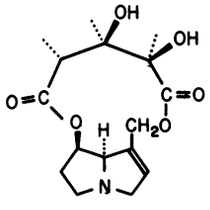
*Contributed by Thomas Eisner, April 13, 1988*

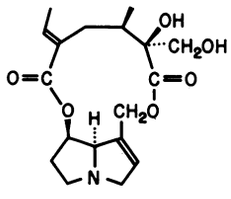
ABSTRACT **The eggs of *Utetheisa ornatrix* contain pyrrolizidine alkaloids. These compounds are contributed by both parents, who sequester them as larvae from their food plants. Females receive alkaloid from the males at mating, apparently by seminal infusion, and transmit this alkaloid together with alkaloid of their own to the eggs. Field and laboratory tests showed that the alkaloids protect eggs from predators. The alkaloidal contribution of the male, although smaller than that of the female, itself provides significant egg protection. A previously identified pheromone, derived by the male from the alkaloid and emitted during precopulatory behavior, may announce the male alkaloidal worth to the female.**

The egg is perhaps the most endangered stage in the life cycle of an insect. Motionless and often conspicuous, it is highly vulnerable to both predators and parasites. Many insects defend their eggs by concealing them, affixing them to stalks, or endowing them with deterrent chemicals (1). As a rule, only the female parent provides for such defenses. Biparental contribution to egg defense is rare in insects (2) and appears unnoted in regard to bestowment of chemical weaponry. We present evidence that in the moth *Utetheisa ornatrix* (family Arctiidae) egg defense is achieved by pyrrolizidine alkaloids, sequestered by the parent insects from their larval food plants, and supplied to the eggs by both sexes.

The larval food plants of *U. ornatrix* are legumes of the genus *Crotalaria*, plants long known to contain pyrrolizidine alkaloids [for example, monocrotaline (compound I) and




I

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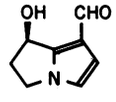


III

usaramine (compound II)]. *Utetheisa* larvae feed preferentially on the seeds of these plants, where the alkaloids are concentrated (3, 4). The larvae tolerate the alkaloids, which they accumulate systemically and retain through metamorphosis into the adult stage. The acquired alkaloid protects both larvae and adults against predation (ref. 5; T.E., unpublished results). Male *Utetheisa* produce a courtship pheromone, hydroxydanaidal (compound III), which they derive chemically from the alkaloid. They secrete the substance from a pair of brush-like structures, the coremata, during their close-range precopulatory interactions with the female. Hydroxydanaidal plays a key role in mediating acceptance of the male by the female. Males reared on a laboratory diet devoid of alkaloid produce no hydroxydanaidal and as a consequence are substantially less successful in courtship (6). This finding led to the suggestion that hydroxydanaidal plays a subtle communicative role. Given its derivation from systemic alkaloid, the pheromone could provide the female with a measure of the male defensive alkaloid load and, hence, with an indirect indication of his larval alkaloid-sequestering ability, a trait potentially heritable (6, 7). We now find this hypothesis restrictive after we discovered that the male transfers some alkaloid to the female at mating as a "nuptial gift" for eventual incorporation into the eggs. Hydroxydanaidal could thus serve directly as a measure of this gift, rather than merely indirectly for assessment of male fitness. Our data are advanced within this conceptual context.

Specifically, we show that (*i*) field-collected eggs of *U. ornatrix* contain pyrrolizidine alkaloid matching that in the natural larval food plants of the moth; (*ii*) eggs parented in the laboratory contain alkaloid contributed by both parents; (*iii*) unmated males contain substantial levels of alkaloid within their reproductive tract; and (*iv*) pyrrolizidine alkaloids effectively protect *Utetheisa* eggs against predators, even in the amount supplied to the egg by the father alone.

## MATERIALS AND METHODS

**Chemical Analyses.** Monocrotaline (compound I) was obtained by extraction of *Crotalaria spectabilis* seeds using a standard procedure (8). Its *N*-oxide was prepared by treating the free base with hydrogen peroxide (9). Alkaloids were isolated from *Utetheisa* parts and eggs using a microscale adaptation of standard protocol (8). Because pyrrolizidine alkaloids occur naturally as both free bases and *N*-oxides, total content of monocrotaline or usaramine in a given sample was measured by reducing any *N*-oxides to the corresponding free base with zinc dust before analysis.

Each sample was extracted for 24 hr at room temperature with methanol. This extract was filtered and evaporated to give a residue that was distributed between 1 M sulfuric acid and chloroform. The acidic aqueous layer was stirred with zinc dust for 3 hr, the zinc was removed by filtration, and the

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Abbreviations: PB, pinto bean; CS, *Crotalaria spectabilis*; CM, *Crotalaria mucronata*.

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filtrate was brought to pH 10 by addition of concentrated ammonium hydroxide. Total alkaloid was then obtained by extraction with chloroform.

In those instances where it was of interest to differentiate between free base and its *N*-oxide, an aliquot of the initial acidic aqueous solution was removed before zinc dust reduction. Basification with ammonium hydroxide, followed by chloroform extraction, yielded only that part of total alkaloid present as the free base. *N*-Oxide content was calculated by subtracting free base titer from total alkaloid content.

Alkaloid samples were quantified by conversion to volatile trimethylsilyl ($Me_3Si$) derivatives, using Supelco Sylon BTZ [bis($Me_3Si$)acetamide/$Me_3SiCl$/$M_3Si$-imidazole, 3:2:3] reagent under standard conditions, followed by gas chromatographic analysis (3% OV-17 or OV-101 on Gaschrom Q; 160–280°C at 4°C/min) using perylene as internal standard. Under our conditions, usaramine was converted into its bis-$Me_3Si$ derivative, whereas monocrotaline yielded a mixture of mono-$Me_3Si$ and bis-$Me_3Si$ derivatives. Consequently, monocrotaline contents were calculated by summing the values for its mono- and bis-derivatives.

**Experimental Animals.** Except where noted, all *Utetheisa* were from our laboratory culture, established with stock taken near Lake Placid and Gainesville, FL. Voucher specimens of adults, which conform in appearance to the subspecies *Utetheisa ornatrix bella* (10), have been deposited (lot 1154) in the Cornell insect collection.

At their original field sites, the *Utetheisa* are found associated with two principal food plants, *Cr. spectabilis* and *Crotalaria mucronata*, differing in pyrrolizidine alkaloid composition. *Cr. spectabilis* contains primarily monocrotaline (compound **I**), and *Cr. mucronata* contains mostly usaramine (compound **II**) (11, 12). We confirmed by analysis of seed pods that neither plant contains the principal alkaloid of the other. In the laboratory, we raised *Utetheisa* on three diets: (*i*) PB diet, a semisynthetic diet based on pinto beans (PB) (13) and devoid of pyrrolizidine alkaloids; (*ii*) CS diet, identical to PB diet, but with *Cr. spectabilis* (CS) seeds in lieu of pinto beans; and (*iii*) CM diet, identical to the preceding diet but with seeds of *Cr. mucronata* (CM) instead of *Cr. spectabilis*. We had shown by analyses that moths reared on PB diet lack pyrrolizidine alkaloid, whereas those raised on CS and CM diet contain monocrotaline and usaramine, respectively. Henceforth, the dietary prefixes will denote the dietary background of adults.

**Alkaloid Content of Field-Collected Eggs.** In the field, *Utetheisa* lay their eggs in clusters on *Crotalaria*, mostly on the underside of leaves. Seven clusters of 13–20 eggs each were taken from a dense stand of *Cr. mucronata* near Lake Placid, FL, at a site where the co-occurring *Cr. spectabilis* was almost totally absent. A sample of five lumped eggs from each cluster was analyzed for alkaloid content (*N*-oxide and free base).

**Maternal and Paternal Alkaloid Contribution to Egg.** To determine the relative alkaloid contribution by each parent to the egg, three individual crosses were effected between males reared on CS diet and females reared on CM diet (3-day-old virgins of both sexes, paired overnight in mating chambers). The males were killed by freezing after mating; the females were provided with honey/water solution and allowed to lay eggs on wax paper until death. Males, females, and the lumped eggs of each female were analyzed for monocrotaline and usaramine content.

**Systemic Alkaloid Distribution in Virgin Adult Males.** To determine whether *Utetheisa* males concentrate pyrrolizidine alkaloid in their reproductive system for transfer during mating, three 4-day-old virgin males (CS diet) were individually dissected into various component parts, including subcomponents of the reproductive system, and each part was analyzed for monocrotaline content.

**Survivorship of Eggs in the Field.** Relative survivorship was determined of *Utetheisa* eggs parented by moths reared on either PB diet or CS diet. (The former eggs could be expected to be alkaloid-free and the latter to contain monocrotaline.) Eggs of each category were collected from cages housing ≈20 adults, where females mated repeatedly, as they do in nature (14). Wax paper in the cages provided the oviposition substrate. Egg clusters were collected daily, adjusted to a standard 10 eggs per cluster (by removing excess eggs), and immediately placed in the field. A total of 100 clusters per category were tested.

The test was done in midsummer near Lake Placid, FL, in a dense stand of *Cr. mucronata* where *Utetheisa* was naturally established. The clusters, each still affixed to a piece (≈2 $cm^2$) of wax paper, were pinned in pairs (one of each category per pair) to the underside of individual *Cr. mucronata* leaves. These leaves are typically trifoliate; paired clusters were consistently pinned to the outer two leaflets of the leaf. After 48 hr a count was taken of the total number of eggs per category that had disappeared (entirely or with only remnants of egg shell remaining). Intact eggs were kept for determination of parasite emergences and viability. Eggs were judged viable if the larvae hatched, died in hatching, or had visibly developed to maturity (head capsule discernable) but failed to hatch. No hatchings occurred during field exposure of the clusters because *Utetheisa* eggs require ≈4 days to mature.

**Degree of Protection Conferred by Each Parent's Alkaloid Contribution to Egg.** A bioassay with a predaceous beetle, *Coleomegilla maculata* (Coccinellidae), was developed for determination of palatability of *Utetheisa* eggs. *Co. maculata* includes lepidopteran eggs in its varied diet (15, 16) and overlaps in range with *U. ornatrix* (10, 17). Our specimens were taken near Ithaca, NY.

Eggs from the following four crosses were offered to the beetle: (*i*) both parents alkaloid-free (PB diet); (*ii*) father usaramine-laden (CM diet) and mother alkaloid-free (PB diet); (*iii*) father alkaloid-free (PB diet) and mother usaramine-laden (CM diet); (*iv*) both parents usaramine-laden (CM diet). Eggs of each category were collected from cages housing groups of adults, as for the field predation test.

For assays, individual beetles in plastic dishes, starved for 1 day, were offered four 10-egg clusters—one from each mating category. Clusters were presented still affixed to squares of wax-paper backing. After a 3-hr feeding period, the beetles were removed from the enclosures, and the eggs remaining in each cluster were tallied as intact, partially eaten (egg contents discernable), or totally eaten (shell remnants at most). Beetles that consumed less than six eggs per test were disregarded. With each beetle the test was repeated, and results were averaged. Two groups of 15 beetles were tested by this protocol. Data from both groups proved comparable and, hence, were lumped.

To relate the unpalatability data to alkaloid load per egg, three samples of 200 eggs from each mating category were assayed for usaramine content. Two additional samples of 200 eggs—one each from mating categories *ii* (CM father) and *iii* (CM mother)—were analyzed to determine the proportion in which usaramine occurs as free base and *N*-oxide.

**Feeding Deterrency of Monocrotaline.** Bioassay with *Co. maculata* allowed direct assessment of feeding deterrency of a pyrrolizidine alkaloid. Of the two *Crotalaria* alkaloids, only monocrotaline was available in sufficient quantity for testing both as *N*-oxide and free base. Prestarved *Coleomegilla* in dishes were again used in discrimination tests with egg clusters. The basic protocol was the same as described except that individual beetles were tested only once. Tests were of 3-hr duration, clusters of 10 eggs were used, and

results were scored as eggs intact, partially eaten, or totally eaten.

The egg clusters were of the following categories: (*i*) experimental—each egg given a topical dose of monocrotaline administered in solution with a microsyringe, either as free base (in methylene chloride) or *N*-oxide (in 95% ethanol); (*ii*) solvent control—each egg treated by topical addition of either methylene chloride (control for free-base treatment) or 95% ethanol (control for *N*-oxide treatment); (*iii*) blank control—all eggs untreated.

The free base and *N*-oxide were each assayed at two doses (0.5 and 1.5 μg per egg; 15 beetles per assay). For each test a beetle was given three egg clusters—one experimental (either free-base or *N*-oxide at one of the dosages), one appropriate solvent control, and one blank control.

## RESULTS

**Alkaloid Content of Field-Collected Eggs.** All seven egg samples contained pyrrolizidine alkaloid of the type (usaramine) found in the parental food plant (*Cr. mucronata*) prevalent at the site where the eggs were collected. Usaramine was present mostly as *N*-oxide. Levels of *N*-oxide per egg, calculated for each five-egg sample and averaged for all egg clusters, was 0.8 ± 0.1 μg ($\bar{x}$ ± SEM; range, 0.5–1.1 μg). Free-base levels, too low for accurate measurement, ranged to estimated maxima of 5% of total alkaloid.

**Maternal and Paternal Alkaloid Contribution to Eggs.** Table 1 shows that eggs contained both monocrotaline and usaramine, indicating that they must have received alkaloid from both parents—monocrotaline from the male and usaramine from the female. The male evidently transmits alkaloid to the female at mating, and the female places some of this alkaloid, together with alkaloid of her own, into the eggs.

The mean quantity of monocrotaline transmitted by the male to the female (66 μg) was 15% of his total load (439 μg). The female transmitted 90% of this gift, together with 90% of her own usaramine, to the eggs. After a single mating, therefore, the male was still substantially endowed with alkaloid. The female, by contrast, after oviposition was mostly depleted. Individually, the eggs received on average 0.4 μg of alkaloid (0.4% of wet weight), of which 32% was contributed by the male. As a result of the male's nuptial gift, total egg output of the female contained over 30% more alkaloid than the total alkaloid initially present in the female.

**Systemic Alkaloid Distribution in Virgin Adult Males.** All major body parts analyzed, including even the wings, contained alkaloid (Table 2). The largest parts, predictably, contained the greatest amounts, but the highest concentration was in the simplex plus aedeagus, a portion of the reproductive system. The simplex [strictly speaking, the *ductus ejaculatorius simplex* (18)] is the long median duct that leads to the intromittent organ (aedeagus). The net quantity of alkaloid in the simplex/aedeagus sample (95 μg) was approximately equal to the quantity of alkaloid of male origin recorded from the body-plus-eggs of females mated with such males (66 μg; Table 1). Evidently, before mating males concentrate alkaloid in their ejaculatory duct for subsequent transfer to females.

Table 1. Alkaloid content of males and females and of their eggs

| | Alkaloid | |
|---|---|---|
| | Monocrotaline, μg | Usaramine, μg |
| Male (after mating) | 373 ± 23 | |
| Female (at death after oviposition) | 7 ± 7 | 15 ± 13 |
| Eggs | 59 ± 5 | 127 ± 29 |
| Total | 439 (= total alkaloid of male origin) | 142 (= total alkaloid of female origin) |

The males were raised on CS diet (monocrotaline-containing), and the females were raised on CM diet (usaramine-containing). Mean ± SEM are given; $n = 3$ mated pairs.

Table 2. Systemic distribution of monocrotaline in virgin male *Utetheisa*

| | Monocrotaline | |
|---|---|---|
| Body part | Total, μg | Wet weight, % |
| Head and thorax | 223 ± 42 | 0.9 |
| Wings | 69 ± 4 | 1.6 |
| Abdomen (minus reproductive system) | 195 ± 19 | 1.1 |
| Reproductive system* | | |
| Testes and vasa deferentia | trace | — |
| Accessory glands | trace | — |
| Duplex | 9 ± 3 | 0.3 |
| Simplex and aedeagus | 95 ± 38 | 3.2 |
| Total | 591 | |

$n = 3$ males reared on CS diet. Mean ± SEM are given.
*Terminology of anatomical components after Drummond (18). The duplex and simplex are subdivisions of the ejaculatory duct.

**Survivorship of Eggs in the Field.** The alkaloid-free eggs (PB parents) showed a significantly higher incidence of disappearance than their alkaloid-laden counterparts (CS parents): 51% versus 28% ($P < 0.001$, Sign test). The occasional presence of shell remnants at the sites of disappeared eggs (84 alkaloid-free versus 9 alkaloid-laden eggs) pointed to the likelihood that mandibulate predators were the primary, if not exclusive cause of the disappearances. Incidence of parasite emergence [mostly a species of *Telenomus* (Hymenoptera)] from the remaining intact eggs was relatively high for both egg categories (37% and 30% for alkaloid-free versus alkaloid-laden eggs). Viabilities of the unparasitized remnant were low (59% versus 35%).

**Degree of Protectedness Conferred by Each Parent's Alkaloid Contribution to the Egg.** The eggs most heavily preyed upon by *Coleomegilla* were those devoid of alkaloid (PB parents) (Fig. 1*A*). Eggs with biparental endowment of alkaloid (CM parents) or with maternal endowment only (CM mother and PB father) showed the highest survival. Eggs endowed by the father only (CM father and PB mother) did not fare as well, but still proved significantly less vulnerable than alkaloid-free eggs (comparison of totally eaten eggs; $P < 0.05$, Sign test).

Eggs left partially eaten are evidence of a beetle's sampling but abandoning such items; *Coleomegilla* clearly showed this behavior most frequently with eggs of biparental or maternal alkaloid endowment. The number partially eaten of the paternally endowed eggs was small but nonetheless significantly greater than that of the alkaloid-free eggs ($P < 0.05$, Sign test).

The palatability data correlated with alkaloid titers in the four categories of eggs (Fig. 1*B*). Eggs of intermediate alkaloid load, endowed by paternal alkaloid only, were also the ones of intermediate palatability.

The alkaloid in eggs occurred predominantly in *N*-oxide form (as in field-collected eggs), irrespective of which parent made the contribution. Eggs from CM fathers contained 0.19 μg of usaramine per egg, 81% as *N*-oxide, whereas eggs from CM mothers contained 0.53 μg of usaramine per egg, 84% as *N*-oxide.

**Feeding Deterrency of Monocrotaline.** Both the *N*-oxide and free base of monocrotaline deterred *Coleomegilla* (Fig. 2). The *N*-oxide at both concentrations and the free base at the higher concentration showed significant deterrency relative to solvent and blank controls ($P < 0.01$, Sign test). Efficacy

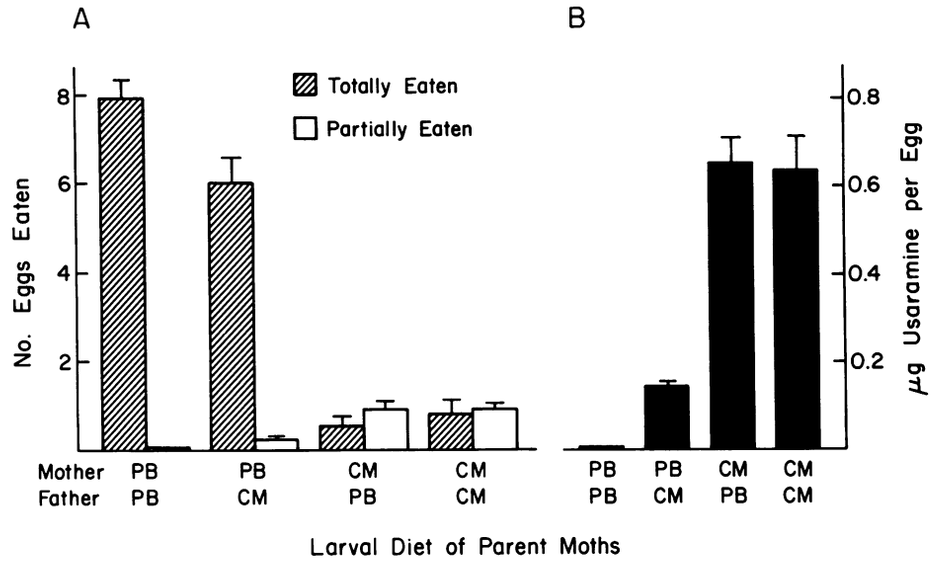


FIG. 1. Acceptability of *Utetheisa* eggs to *Co. maculata* (*A*) and usaramine content of eggs (*B*), plotted as a function of larval diet of parent moths. Mean and SEM are given. For *A* $n = 30$ beetles, and for *B* $n$ = three samples of 200 eggs per category.

of the free base at the lower concentration (0.5 μg) did not differ from the solvent control.

Beetles tested with *N*-oxide and its controls consumed less eggs overall. Perhaps these beetles were less voracious because they were tested in midwinter after prolonged laboratory refrigeration.

## DISCUSSION

Two principal points seem established: the pyrrolizidine alkaloid in the eggs of *Utetheisa* is of biparental origin, and this alkaloid serves for defense. The female provides the larger fraction of the egg alkaloid, but the male's contribution represents a significant addition. Eggs endowed by paternal alkaloid only, from mothers that were alkaloid free, proved less edible to *Coleomegilla* than eggs totally devoid of alkaloid. One might anticipate, therefore, that females should mate selectively with males able to bestow alkaloidal gifts because these can be invested in egg defense and that the males should have the means for advertising their alkaloid-donating capacity to the female.

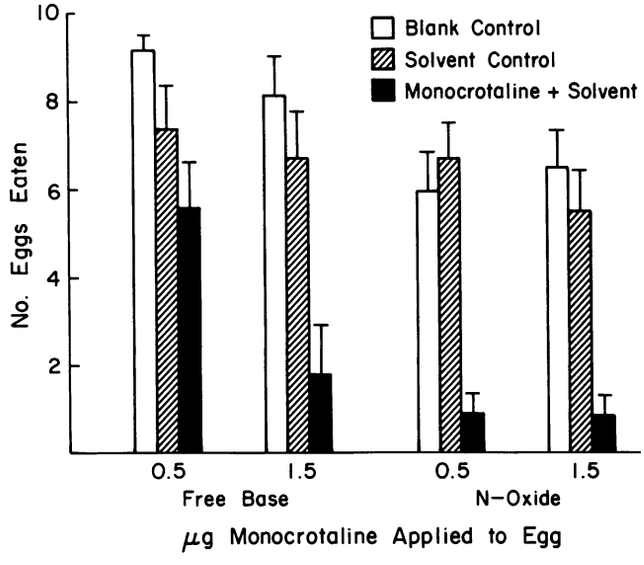


FIG. 2. Acceptability of *Utetheisa* eggs to *Co. maculata*, plotted as a function of monocrotaline dosage (free base and *N*-oxide) topically applied to egg. Mean and SEM are given; $n = 15$ beetles per category.

We know from previous work that *Utetheisa* females favor males that secrete hydroxydanaidal, the courtship pheromone derived by the males from systemic alkaloid (6). This pheromone, we suggest, is the male announcement of his worth. Data to be published elsewhere show that the male secretes hydroxydanaidal in approximate proportion to systemic alkaloid content, which itself is proportional to the fraction of alkaloid transmitted in mating (19). The pheromone could thus provide the female with a measure of a male's potential nuptial gift. Female *Utetheisa* can effect such assessment: their antennae bear chemoreceptors highly sensitive to hydroxydanaidal (20).

In nature, female *Utetheisa* often mate repeatedly [as many as 11 spermatophores have been detected in single females (14)], and they could therefore invest more paternal alkaloid in eggs than the relatively small amounts received from single matings. For females of low intrinsic alkaloid content, who as larvae might have fed predominantly on leaves rather than the alkaloid-rich seeds of the food plant, receipt of multiple alkaloidal gifts could be of decisive importance. Questions arise also about male strategy. Do males dispense alkaloids relatively sparingly at mating as a matter of course? If so, do they retain alkaloids to preserve their own defense or for donation to additional females? Further, do females allot their multiple alkaloidal gifts in admixture to the eggs or as individual donations to consecutive batches of eggs? Is the male donation transmitted to eggs of his siring?

Such uncertainties notwithstanding, the field data pertinent to the eggs and their fate *vis à vis* predators support the laboratory findings. Alkaloid levels in field-collected eggs and in eggs from our experimental matings proved comparable. Eggs devoid of alkaloid disappeared faster when exposed outdoors than alkaloid-laden counterparts, just as such eggs proved more acceptable to *Coleomegilla* in laboratory tests. Clearly, the unacceptability of alkaloid-laden eggs is attributable to the alkaloid itself. Experimental addition of monocrotaline to alkaloid-free eggs in amounts commensurate with natural alkaloidal endowments, rendered the eggs relatively unacceptable to *Coleomegilla*.

The mating and egg-investment strategy exemplified by *Utetheisa* has remarkably close parallels in other insects. A number of Lepidoptera sequester pyrrolizidine alkaloids from plants, either as larvae or adults, and produce eggs that

(in at least some cases) themselves contain such alkaloids (19, 21–25). Males in some of these species also derive courtship pheromones from the ingested alkaloid (25, 26). In danaine butterflies, for instance, adult males and, to a lesser extent, females visit pyrrolizidine alkaloid-containing plants to imbibe fluids from damaged or senescent parts thereof (26–28). The pheromone that the males derive from the acquired alkaloid has been shown in one species, the queen butterfly *Danaus gilippus*, to mediate their acceptance in courtship (29). In that same species we have now demonstrated that the male passes much of his ingested alkaloid to the reproductive system for eventual copulatory transfer to the female and that The female in turn transmits the alkaloid to eggs (19). In ithomine butterflies, a group closely related to danaines, the alkaloid in eggs appears also to be of paternal origin (22, 23). In such cases, where the female herself may contribute little or no alkaloid to the eggs, one would presume her to be particularly discriminating in courtship.

Some insects employ compounds of endogenous origin, rather than plant metabolites, for egg defense. Meloid beetles, for example, endow their eggs with cantharidin, the very agent that protects the adult. In some meloids, the egg derives its cantharidin from the male, which synthesizes the compound and transfers it to the female at mating (ref. 30; J. C. Carrel and T.E., unpublished data).

Parental investment of egg defenses will doubtless prove of widespread occurrence, at least in insects, and may involve a broad array of metabolites. We predict, moreover, that female choice mechanisms, functionally comparable to those in *U. ornatrix* and *Danaus gilippus*, will probably be found as concomitants of the strategy wherever males contribute significantly to the investment.

We are indebted to B. L. Roach of our Cornell laboratories for the alkaloid analyses by means of high-pressure liquid chromatography of the field-collected *Utetheisa* eggs. We also thank W. E. Conner, M. Achey, D. Andow, and I. Baldwin for helpful discussion; J. Gillmore, D. H. Habeck, and R. L. Rutowski for supplying field-collected *Utetheisa*; E. Jakob and J. Boggan for technical help; S. Shaw (United States Department of Agriculture Systematic Entomology Laboratory) for identifying *Telenomus*; and the staff of the Archbold Biological Station for help and hospitality. Study supported by Grants AI02908 to T.E. and AI12020 to J.M., a Predoctoral Traineeship (to D.E.D.) from the National Institutes of Health, and by grants from the Sigma Xi (to D.E.D.).

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