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Chemical basis of courtship in a beetle (*Neopyrochroa flabellata*): Cantharidin as "nuptial gift"*

(sexual selection/parental investment/pheromone/Spanish fly/Coleoptera: Pyrochroidae)

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ABSTRACT **The amount of cantharidin (Spanish fly) that the *Neopyrochroa flabellata* male presents to the female as a glandular offering during courtship represents only a small fraction of the total cantharidin the male accumulates systemically following ingestion of the compound. A major fraction of the acquired cantharidin is stored by the male in the large accessory glands of the reproductive system. At mating, the male transfers this supply, presumably as part of the sperm package, to the spermatheca of the female. The female in turn allocates the gift to the eggs. Eggs endowed with cantharidin proved relatively invulnerable to attack by a predaceous beetle larva (*Coleomegilla maculata*).**

We demonstrate here that the cantharidin ingested by male *Neopyrochroa flabellata* (1) is transferred in large measure to the female at mating, and by the female, for protective purposes, to the eggs. Specifically, we demonstrate that (*i*) cantharidin (Spanish fly), ingested by the male, accumulates primarily in the large accessory glands of the reproductive system; (*ii*) mating leads to appearance of cantharidin in the sperm receptacle (spermatheca) of the female; (*iii*) eggs sired by cantharidin-fed males contain cantharidin; and (*iv*) cantharidin-laden eggs, unlike cantharidin-free eggs, are protected against predation. Preliminary aspects of this study were reported earlier (2).

MATERIALS AND METHODS

Source and maintenance of beetles, chemical analyses for cantharidin content, and statistical analyses were as described (1). Values (including those in the figures) are given as mean ± SEM.

Cantharidin Feeding. Males designated as cantharidin-fed (n = 58) were offered crystalline cantharidin as described (1). Total cantharidin offered to individuals ranged from 5 to 3050 μg, given over a span of 1–31 days. Mean quantity per beetle was 766 ± 83 μg, given over a period of 8.4 ± 0.9 days.

Males designated as cantharidin-unfed (n = 34) were kept unexposed to cantharidin.

Dissection. Beetles were killed by freezing and dissected under saline solution. Components of the male reproductive system that were analyzed for cantharidin content were (see Fig. 1*A*) as follows: testes, including the ducts leading to the seminal vesicles; seminal vesicles; large accessory glands; small accessory glands; and ejaculatory duct. Components of the female reproductive system that were analyzed were (see Fig. 1*C*) as follows: ovaries, spermatheca, and median oviduct. For both males and females, heads were also analyzed, as well as the alimentary canal, and a sample, designated as remains, consisting of all body parts, minus head, reproductive system, and gut. All samples were weighed immediately after dissection. A small fraction of samples was lost in the course of the analyses (sample sizes for component parts were therefore sometimes variable).

Dissection of mated males (n = 7) and mated females (n = 9) was performed, respectively, within 1.0–3.5 h (2.6 ± 0.4 h) and 0.1–2.7 h (1.2 ± 0.3 h) after mating.

Matings. These were staged in Petri dishes, as in the courtship trials described (1). All females were virgin at the time of mating.

Cantharidin Allocation to Eggs. To determine whether cantharidin is allocated to the eggs, the cantharidin content of eggs sired by cantharidin-fed males (n = 80 egg samples from 19 singly mated females) was compared with that of eggs sired by cantharidin-unfed males (n = 41 egg samples from 12 singly mated females). For each egg sample, the number of contained eggs (72.2 ± 4.7 eggs; range, 6–231) as well as the oviposition date (relative to day of mating) was recorded, permitting calculation of the mean cantharidin content per egg laid at various times past mating. The postmating oviposition span was arbitrarily divided into five 3-day periods (1–3, 4–6, 7–9, 10–12, and 13–15 days past mating) and a final longer period (16–36 days past mating). If for a given period more than one egg sample from a particular female was analyzed, then their averaged cantharidin value was entered as the value for that female in the calculation of the overall mean for the period.

To estimate the total ovipositional cantharidin output of females (n = 12 females singly mated to cantharidin-fed males), a mean was determined for the total eggs produced by these females during each of the six oviposition periods. Each of these values was multiplied by the mean egg cantharidin content for that period, and the six resulting products were summed.

Egg Predation Tests. These were staged in small Petri dishes [same type as used in courtship trials (1)] and involved offering four pairs of eggs to an individual predator, two pairs sired by a cantharidin-fed male, and the other two (controls) sired by a cantharidin-unfed male. The eggs were presented on a small coverslip (18 mm square), in such arrangement that the pairs of similar type were at opposite corners of the coverslip. The predators were nearly full grown larvae of the coccinellid beetle *Coleomegilla maculata*. Tests were of 60 min duration, during which the fate of the eggs (whether eaten, partially eaten, or left uneaten) was noted. Also recorded was the number of times that eggs of either type were rejected (larva turned away) after inspection (contact with mouth parts). Twelve replicate tests were undertaken, each with a different larva.

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RESULTS

Male and Female: Control Whole Body Cantharidin Determinations. Analyses of whole bodies of cantharidin-unfed males (n = 21) showed such samples to contain no detectable levels of cantharidin. Virgin females (n = 6; physically unexposed to males) were likewise found to be cantharidin-free (four of the females were analyzed as whole body samples; two were dissected into component parts, which all proved to be cantharidin-free).

Male and Female Reproductive Systems. Anatomically these conform to the basic plan characteristic of Coleoptera. In the male (Fig. 1*A*), sperm are produced by the testes, conveyed to the seminal vesicles, and expelled at mating through the ejaculatory duct. A pair of large accessory glands and a pair of small accessory glands open jointly into the base of the ejaculatory duct. Whereas prior to mating (Fig. 1*A*) the large accessory glands and seminal vesicles were noted to be turgid with contained whitish material, both structures were empty and translucent after mating (Fig. 1*B*).

In the female (Fig. 1*C*), the eggs are produced in the ovaries and conveyed from these by short ducts to the median oviduct through which they are laid. The median oviduct also serves for admission of the sperm, which are stored within the diverticular spermatheca. Before mating (Fig. 1*C*), the spermatheca was noted to be translucent and empty; after mating (Fig. 1*D*) it was filled to capacity with the male's encapsulated sperm package (spermatophore).

Based on the difference in mass of the spermatheca before mating (1.80 ± 0.32 mg; n = 10) and after mating with cantharidin-fed males (10.31 ± 0.78 mg; n = 9), we calculate the mass of the spermatophore to be on average about 8.5 mg, or about 10% of male body mass (Table 1). This figure matched closely the whole body mass loss of cantharidin-fed males at mating (9.19 ± 0.46 mg; n = 22) and the whole body mass gain of their female partners (8.28 ± 0.46 mg; n = 22).

Male: Systemic Distribution of Ingested Cantharidin. Cantharidin-fed males proved to have an uneven body distribution of cantharidin (Fig. 2*A*, solid columns). Maximal net amounts of the chemical were detected in the head, remains, and large accessory glands. Other body parts contained distinctly lesser amounts. Concentration of cantharidin was highest, and next to highest, in the large accessory glands and head, respectively. Both these structures were of lesser mass, by a factor of 10, than the remains (Table 1), yet contained as much, and almost a third as much, cantharidin as the remains. The large accessory glands, in effect, although amounting to less than 5 mg (≈5% of body mass) contained fully 39% of the total body cantharidin of the male. We attribute the relatively high level of cantharidin in the head to the presence of the cantharidin-secreting gland (1).

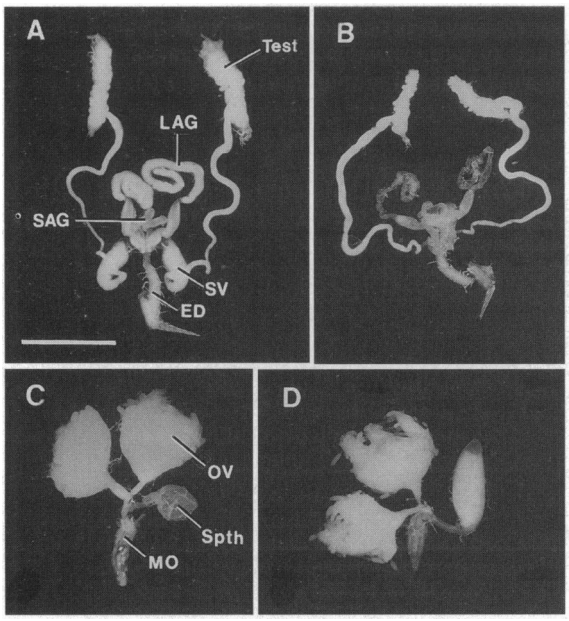

FIG. 1. *N. flabellata* reproductive systems. (*A*) Male, before mating (Test, testes; SV, seminal vesicle; SAG, small accessory glands; LAG, large accessory glands; ED, ejaculatory duct). (*B*) Male, after mating. (*C*) Female, before mating (OV, ovary; MO, median oviduct; Spth, spermatheca). (*D*) Female, after mating. (Bar = 5 mm.)

Table 1. Mass per whole body and body parts

	Male (n = 25)	Female (n = 9)
Whole body	78.20 ± 3.22	135.46 ± 6.51
Head	4.15 ± 0.12	4.31 ± 0.34
Rems	54.66 ± 2.00	63.50 ± 3.72
Gut	2.47 ± 0.21	2.97 ± 0.37
Test	3.14 ± 0.48	
SV	3.38 ± 0.30	
LAG	4.30 ± 0.34	
SAG	0.84 ± 0.14	
ED	1.43 ± 0.08	
OV		44.30 ± 3.50
Spth		10.31 ± 0.78
MO		1.94 ± 0.22

Rems, remains; Gut, alimentary canal; Test, testes; SV, seminal vesicles; LAG, large accessory glands; SAG, small accessory glands; ED, ejaculatory duct; OV, ovaries; Spth, spermatheca; MO, median oviduct.

Male: Cantharidin Loss at Mating. Comparison of the solid and hatched columns in Fig. 2*A* shows that mating resulted in partial loss of cantharidin in the male. The body parts that underwent the greatest amount of cantharidin depletion were those initially most richly endowed with the compound: the head, the remains, and the large accessory glands. Net loss from the accessory glands alone amounted to ≈17 μg. Combined loss of accessory glands and remains was ≈35 μg.

Female: Cantharidin Gain at Mating. Cantharidin in the newly mated female was present mostly in the spermatheca (Fig. 2*B*). The total amount present in this receptacle (≈25 μg) amounted to about 75% of the total cantharidin acquired by the female.

Cantharidin Allocation to Eggs. As is evident from Fig. 3, eggs sired by cantharidin-fed males contained cantharidin, while eggs sired by cantharidin-unfed males (with one exception) were cantharidin-free (we attribute the exception, which was due to two aberrant cantharidin values in the 16- to 36-day category, to experimental error).

It is clear that females did not allocate cantharidin evenly to the eggs over time after mating. Rather, they allocated cantharidin in increasing quantities until days 10–12, and then in decreasing amounts until death. Even their last-laid eggs contained at least some cantharidin.

Total cantharidin output by way of the eggs (n = 12 females) was estimated (see *Materials and Methods*) to be 13.0 μg per female.

Neither female fecundity (number of eggs laid over life span) nor egg mass was affected by whether the sire was cantharidin-laden. For females mated with cantharidin-fed males (n = 12 females), fecundity was 592 ± 112 eggs per female, while for females mated with cantharidin-unfed males (n = 6) it was 555 ± 116 eggs per female (t test, P = 0.82). Egg mass for females of the former category was 59.7 ± 1.1 μg (based on 12 egg batches from separate females), while for those of the latter category it was 59.3 ± 2.5 μg (based on 8 egg batches from separate females) (t test, P = 0.90).

Egg Predation Tests. The cantharidin-laden eggs proved distinctly less vulnerable *vis à vis C. maculata* larvae than the

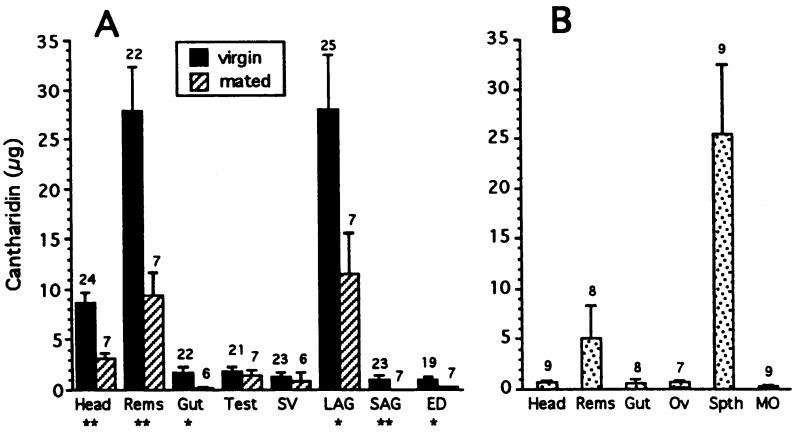

FIG. 2. (*A*) Cantharidin content of body parts of cantharidin-fed *N. flabellata* males (virgin and mated) (*, $0.01 \leq P < 0.05$; **, $P < 0.01$; *t* tests). (*B*) Cantharidin content of females mated with cantharidin-fed males. Numbers above columns give sample sizes; body part abbreviations are defined in Fig. 1 and as follows: Rems, remains; Gut, alimentary canal.

cantharidin-free eggs (Fig. 4*A*). Moreover, cantharidin-laden eggs were often rejected on contact by the larvae, unlike their cantharidin-free counterparts (Fig. 4*B*).

DISCUSSION

The sequence of events which in *N. flabellata* lead from acquisition of cantharidin to allocation of the chemical to the eggs is schematically depicted in Fig. 5. Our data show that the male incorporates systemically some 70 μg of the cantharidin he ingests and that he transmits about 40 μg of this load to the female at mating. The female in turn allocates the gift to the eggs, at a dosage (on average) of 23 ng/egg. Her total cantharidin output by way of the eggs (≈13 μg) amounts to ≈33% of the cantharidin she receives from her mate. The overall "flow" of cantharidin, from male-to-female-to-eggs, therefore occurs with considerable efficiency.

The quantity of cantharidin gained by the female at mating is closely matched by the amount held by the male in the large accessory glands before mating. We postulate that it is from these glands that the female receives her seminal gift of cantharidin, and that the glands give up their entire cantharidin supply at mating. Yet we found the glands of mated males to be only partly cantharidin-depleted, and the bodies of these males (that is, the body "remains") also to be cantharidin-impoverished. We attribute this result to our having dissected these males after a delay rather than immediately following mating, a delay that could have sufficed for the large accessory glands to begin reacquiring cantharidin from the body reserves. Indeed, the amount of cantharidin lost by the "remains" of mated males was an approximate match of the amount present in the large accessory glands of these individuals. We conclude that the large accessory glands reload with cantharidin relatively quickly after copulation.

Whether males are able to mate again shortly after having mated remains unknown. We know from laboratory tests that males are able to mate at intervals of days, and that individuals can mate up to seven times over their life span. The females are themselves able to mate more than once, but they are incapable of physically accommodating a second spermatophore until at least several days following a previous mating. Since during this period a female can lay a substantial number of eggs, males mating with virgins have assurance of siring at least a fraction of the offspring they endow with cantharidin. Whether males mating with non-virgins have similar assurance remains unknown, given that the details of sperm competition have yet to be elucidated for *N. flabellata*.

Such evidence as we have indicates that cantharidin is added to the eggs in the ovary rather than directly from the spermatheca as the eggs descend along the median oviduct on the way to being laid. Ovaries dissected from females that had mated with cantharidin-laden males weeks beforehand, and had oviposited for many days, contained substantial levels of cantharidin (3.9 ± 0.9 μg; $n = 12$). The fact that eggs first laid

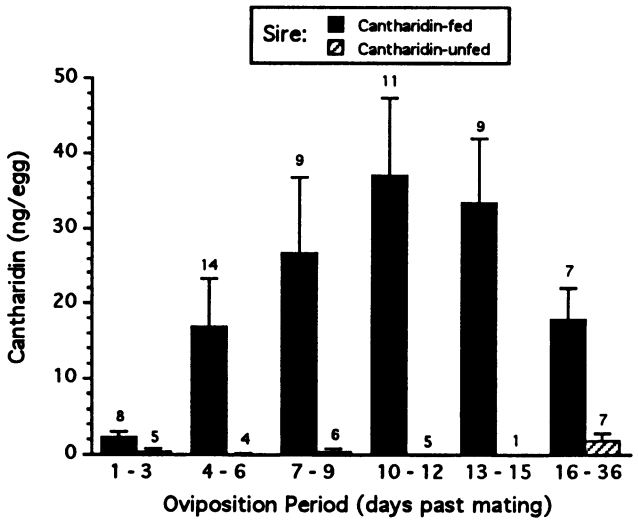

FIG. 3. Cantharidin content of *N. flabellata* eggs plotted as a function of cantharidin status of sire and time of deposition. Numbers above columns give sample sizes.

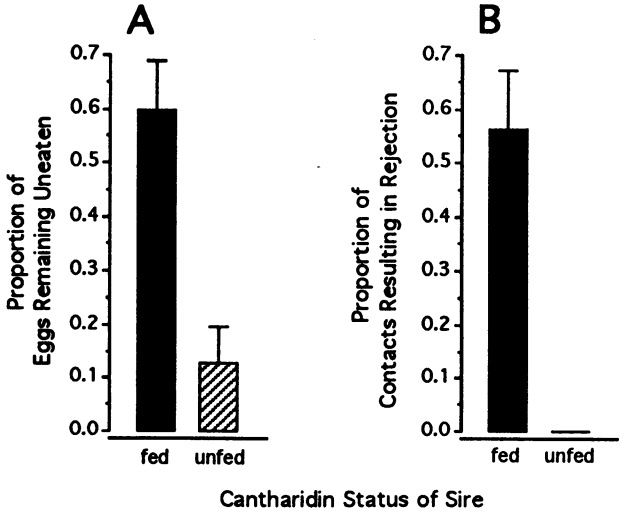

FIG. 4. Results of predation tests with *C. maculata* larvae. (*A*) Fate of the two types of *N. flabellata* eggs (proportion of original eggs remaining uneaten per trial). (*B*) Response of predator to contact with these eggs (proportion of total number of contacts resulting in rejection per trial). In both cases, $P \leq 0.01$, Wilcoxon-signed rank tests ($n = 12$ pairs).

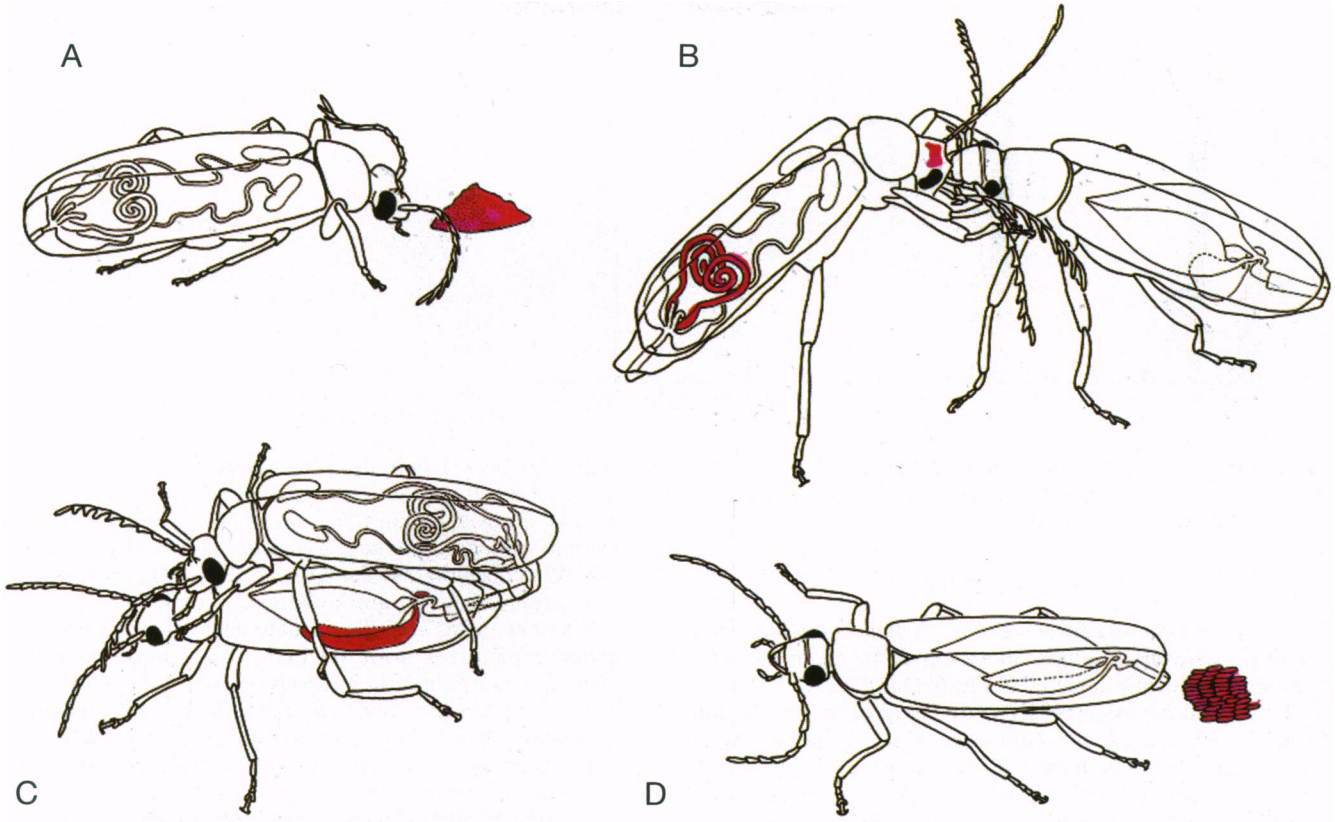

FIG. 5. Use of cantharidin by *N. flabellata*. The male procures the chemical (*A*), and after ingestion stores it in the cephalic gland and in the large accessory glands of the reproductive system. The female samples secretion from the male's cephalic gland in courtship (*B*), and as a sequel yields to the male's copulatory attempts. The male inseminates the female and in the process transfers cantharidin from his accessory glands to the female's spermatheca (*C*). The female in turn bestows the acquired cantharidin upon the eggs (*D*).

after mating are not fully endowed with cantharidin, itself suggests that the pathway of conveyance of the chemical, from spermatheca to eggs, is a circuitous one. We presume the ovarial addition of cantharidin to occur while the eggs are in the developmental stage and not as yet encased in shells. Such early chemical endowment could ensure that the eggs are impregnated with cantharidin rather than merely coated topically with the substance.

We presume the cantharidin in the eggs to protect against predators other than coccinellid larvae as well. Prime enemies, for instance, could be wood-inhabiting ants. Interestingly, ants have been shown to be orally deterred by as little as 10^{-5} M cantharidin (3). We calculate the concentration of cantharidin in *N. flabellata* eggs to be in the order of 10^{-2}–10^{-3} M. Additional enemies that might be deterred by cantharidin include carabid beetles [which are also highly cantharidin-sensitive (3)], mites, centipedes, and parasitoids. One wonders also whether cantharidin could protect eggs against microbial infection.

Conceivably even the adults could derive protection from their acquired cantharidin. The males, certainly, could benefit, given that they even externalize some of the compound by way of the cephalic secretion. But females could themselves be deterrently "labeled" as a consequence of receipt of their nuptial gift.

We presume the spermatophore of the male, given that it amounts to ≈10% of male body mass, to contain a substantial quantity of nutrient besides cantharidin and sperm. It would not be unusual for an insect spermatophore to be so endowed. Conveyance of nutrient by way of the spermatophore has been demonstrated for a number of insects, and female fecundity has been shown to be increased as a result (4–7).

The basics of the reproductive strategy of *N. flabellata* seem established, but some questions remain unanswered. Do females discriminate between males strictly on the basis of their possession or nonpossession of cantharidin, or are they able to discern differences in magnitude of cantharidin possession in males? If able to do the latter, do females assess a male's "worth" by the amount of cantharidin he offers with the cephalic secretion? And is this offering a true measure of the cantharidin the male holds in store for copulatory transmission to the female? We have preliminary evidence indicating that there may indeed be a proportionality between the amount of cantharidin a male ingests and the quantity he stores in his cephalic gland.

Cases where eggs are protected by paternal provisioning of defensive chemicals have been previously documented for insects. In certain butterflies and arctiid moths, the males bestow pyrrolizidine alkaloids upon the eggs. The males procure the chemicals from plants, transmit them by seminal infusion to the female, and the female passes them on to the eggs. The eggs are protected as the result (8–10). As in *N. flabellata*, the males of these lepidopterans "inform" females, by use of a pheromone in courtship, that they are endowed with defensive chemical. In their case, unlike in *N. flabellata*, the pheromone is not the defensive chemical itself, but a derivative thereof, a volatile substance that the males produce at the expense of a fraction of their systemic alkaloid (8, 9, 11). The pheromone varies somewhat in structure in these lepidopterans (8), but its message appears consistently to be one of advertisement. In both a danaine butterfly (12) and an arctiid moth (13), it has been shown that males devoid of alkaloid, and therefore unable to produce the pheromone, fare relatively poorly in courtship.

Of some interest is the question of where *N. flabellata* males obtain their cantharidin. Blister beetles (Meloidae) and false blister beetles (Oedemeridae) produce cantharidin (14, 15), but *N. flabellata* males are not known to be predaceous on adult insects. Eggs of meloids and oedemerids contain cantharidin (14–16), but meloid females generally lay their eggs selectively in habitats that would not make them likely resources for *N. flabellata*. Females of several oedemerid species oviposit in wood, but it seems unlikely that this could constitute a common or abundant cantharidin resource. *N. flabellata* males could feed upon the remains of dead adult meloids and oedemerids, but oedemerids are rarely common and meloids are rarely found in forests where *N. flabellata* occurs. Whatever the source of the chemical, we know that *N. flabellata* does obtain cantharidin in nature: two females that we collected in the field and analyzed for cantharidin content (whole body analysis) proved to contain the chemical (identification confirmed by mass spectrometry). *N. flabellata* is not the only insect known to be cantharidiphilic. As noted by many investigators, a number of other beetles, chiefly Anthicidae, Endomychidae, and other Pyrochroidae, as well as certain Diptera, Hemiptera, and Hymenoptera, are also drawn to cantharidin (or to blister beetles) (16–21). In some of these species both sexes are attracted, but in others, it is only the males. We predicted, based on our unfolding results with *N. flabellata*, that cantharidin in these insects might also be used for sexual-selective or egg-protective purposes (2). Indeed, it has now been shown for an anthicid (*Notoxus monocerus*), that the male presents the female with a secretory cantharidin offering during courtship (22), and for a pyrochroid (*Schizotus pectinicornis*), that the eggs receive their cantharidin, at least in part, from the male (16).

One wonders whether cantharidiphilic insects might pose a threat to one another. Might such insects prey on eggs, such as those of *N. flabellata*, that are "protected" by cantharidin? We were curious, in this connection, whether *N. flabellata* males cannibalize eggs. Preliminary tests showed that they ignored conspecific eggs, whether these were cantharidin-laden or cantharidin-free.

Remarkably, in meloid beetles, the cantharidin in the eggs may also stem from the male, which transmits the chemical to the female at mating (14, 23, 24). The strategy is comparable to that of *N. flabellata*, except that in meloids the cantharidin is synthesized by the male itself. A pheromone, such as might inform the female of the cantharidin content of the male, has so far not been discovered in Meloidae.

A final point concerns the broader implications of this study. Insects are the dominant animals on land, and they could owe their success in part to the chemical defenses of their eggs. The substances they use for egg protection could be enormously variable and to a large extent new. We anticipate that a comparative chemical investigation of insect eggs could uncover substances of medicinal and other uses.

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