

## THE LETHAL EFFECTS OF *CYPERUS IRIA* ON *AEDES AEGYPTI*

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**ABSTRACT.** The sedge *Cyperus iria*, a common weed in rice, contains large amounts of the insect hormone (10R) juvenile hormone III (JH III). Given its widespread distribution in Asia and Africa, we examined the possibility that *C. iria* could be used as a safe, inexpensive, and readily available mosquito larvicide. Plants of varying ages were harvested and leaves tested for lethal effects on larvae of the yellow fever mosquito, *Aedes aegypti*. The median lethal doses (LD<sub>50</sub>s) for frozen leaves from 1- and 2-month-old plants were 267 and 427 mg/100 ml of water, respectively. Leaves from 1-month-old *C. iria* contained 193 µg JH III/g fresh weight, whereas leaves from 2-month-old plants contained 143 µg JH III/g fresh weight. Larval sensitivity to the plant differed with age; 4-day-old larvae displayed the greatest mortality followed in decreasing sensitivity by larvae 5, 6, 3, and 2 days old. Six *Cyperus* species (*C. albostriatus*, *C. alternifolius*, *C. esculentus*, *C. iria*, *C. miliifolius*, and *C. papyrus*) of similar developmental stage were assayed for JH III content. Only *C. iria* was found to contain significant levels of JH III.

**KEY WORDS** Juvenile hormone, mosquito, sedge, biological control, larvicide

### INTRODUCTION

Mosquito-borne pathogens infect more than 600 million people annually (Kolberg 1994). Efforts to control mosquitoes and the pathogens they vector are hampered by resistance to insecticides and chemotherapeutic compounds (Kolberg 1994). New approaches to combat mosquito-borne diseases, such as genetically engineered mosquitoes and vaccines, are under intense study but are far from implementation (Spielman 1994, Collins and Paskewitz 1995). Thus, interim strategies are needed to bridge the gap between older methods and future alternatives.

Methoprene, a synthetic juvenile hormone mimic, is an effective mosquito larvicide (Floore et al. 1990, Kramer and Beesley 1991, Knepper et al. 1992, Nasci et al. 1994), displaying low toxicity towards mammals (Garg and Donahue 1989) and most nontarget invertebrate species (Miura and Takahashi 1973). Moreover, the persistence of synthetic juvenile hormone analogs (JHAs) is short, making them ideal for use in environmentally sensitive areas. However, the relatively high cost of JHA control compared with conventional larvicides prevents its use in developing regions. The discovery that a common weed, *Cyperus iria*, contains large quantities of juvenile hormone III (JH III) (Toong et al. 1988) suggested an alternative strategy for mosquito control. This sedge is a ubiquitous weed in rice fields in southeast Asia and disturbed soils in western Africa and the Americas (Holm et al. 1977, Akobundu and Agyakwa 1987). The present study determined whether endogenous JH III in *C. iria* leaves could disrupt mosquito development. Simple processing of the leaves of this plant may

offer an effective, inexpensive, and environmentally safe method of mosquito control.

### MATERIALS AND METHODS

**Cyperus Plants:** Wild *C. iria* seeds obtained from Penang, Malaysia (provided by Y. C. Toong) were planted in soil consisting of 2:1 loam:sand and watered every other day. Plants were grown in a greenhouse under a 16:8 light:dark (L:D) cycle. Harvested leaves were wrapped in aluminum foil that had been washed with methanol and then frozen at -80°C. Unless noted otherwise, all leaf material was frozen until bioassayed. *Cyperus albostriatus*, *C. alternifolius*, and *C. miliifolius* were provided by John Wirth of Olbrich Botanical Gardens, Madison, WI. *Cyperus papyrus* and *C. esculentus* (common name, yellow nutsedge) seeds were obtained from Hawaii and Madison, WI, respectively.

**JH III:** (10R) Juvenile hormone III was extracted from *C. iria* leaves using the method of Toong et al. (1988). The hormone was further purified by high-performance liquid chromatography on a SPHERISORB silica column (LDC, Riviera Beach, FL) (5 µm; 4.6 mm × 25 cm) using the method of Goodman and Adams (1984). The chemical nature of JH III was confirmed by gas chromatography-mass spectrometry using a Shimadzu GC-14a gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a DB-1 (J&W Scientific Co., Folsom, CA) capillary column (30.0 × 0.25 mm, bonded phase) mated to a Finnegan MAT 800 series (Finnegan, San Jose, CA) ion trap detector. The gas chromatograph was temperature-programmed from 10°C/min at 60°C to 280°C; both the injection port and the transfer line were maintained at 250°C.

**Radioimmunoassay:** Juvenile hormone III was extracted by cutting the leaf into 15 1 × 2-mm sections (approximately 1.7 mg each), which were then wrapped in 3 × 3-cm sheets of aluminum foil. The foil was prewashed with methanol and treated with polyethylene glycol (1% in water; molecular

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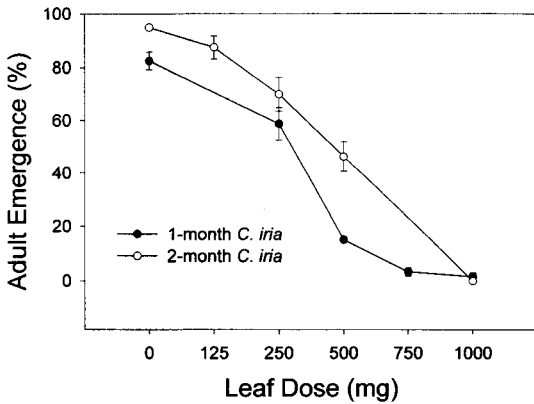


Fig 1. Effect of 1- and 2-month-old *Cyperus iria* on adult emergence of *Aedes aegypti*. Twenty larvae were reared in 100 ml of water, 4 bowls per treatment. Chopped *C. iria* leaves were added to the water at day 4 of larval development. When pupae appeared, the bowls were placed individually in cages. Successful emergence was determined by the mosquitos' ability to fly from the bowl. Emergence was monitored for 3 days after the last control animal emerged. Controls consisted of bowls with no plant material or 1,000 mg *Cyperus esculentus* (data not shown). All leaves used in this study were from frozen stock. Vertical bars represent SE. Absent SE bars indicate that SE was smaller than datum point.

weight 20,000; Sigma Chemical Co., St. Louis, MO) to enhance JH recovery. The foil packet containing the leaf was placed in a ceramic mortar, covered with liquid nitrogen, and the leaf was pulverized with a pestle. The powdered leaf was added to a 30-ml glass centrifuge tube. The foil was quickly fitted to the inside lip of the centrifuge tube and 3 ml of acetonitrile (60°C) was pipetted over the foil to remove the remaining plant material. Except where noted, the standard radioimmunoassay (RIA) protocol was followed (Goodman et al. 1995). [<sup>3</sup>H]Juvenile hormone III (10 Ci/mmol; NEN Research Products, Boston, MA) was added to selected samples to follow recovery. Juvenile hormone antisera 676 and 876 were used at dilutions of 1:4,000 and 1:1,000, respectively (Goodman et al. 1995).

**Mosquitoes:** *Aedes aegypti* (L.) (Rockefeller strain) were reared at 24°C (±3°C) on a 14:10 L:D cycle. Eggs were hatched by placing them under a vacuum for 1 h. Days are referenced from time of hatch.

**Dose-response studies:** Twenty 3-day-old larvae were added to 150-ml Pyrex bowls containing 100 ml of double-distilled water and varying amounts of chopped *C. iria* leaves (4 replicates per leaf treatment). Control bowls contained either 1,000 mg *C. esculentus* or no leaves (4 replicates per leaf treatment). Preliminary assays indicated no statistical difference between populations reared at 10, 15, or 20 larvae/100 ml H<sub>2</sub>O. Near the time of adult emergence the bowl was placed in a 3-gallon cage,

Table 1. Juvenile hormone III (JH III) titers in mature *Cyperus* leaves.

| <i>Cyperus</i> species             | µg JH III/g leaf   |
|------------------------------------|--------------------|
| <i>C. albostratus</i>              | 0.0                |
| <i>C. alternifolius</i>            | 0.0                |
| <i>C. esculentus</i>               | 0.0                |
| <i>C. miliifolius</i>              | 0.0                |
| <i>C. papyrus</i>                  | 0.0                |
| <i>C. iria</i> (3-month-old plant) | 151.0 <sup>1</sup> |
| <i>C. iria</i> (2-month-old plant) | 142.9 ± 9          |
| <i>C. iria</i> (1-month-old plant) | 192.8 ± 5          |

<sup>1</sup> Toong et al. 1988.

covered with nylon mesh. Successful emergence was determined by the adults' ability to fly from the bowl. The assays were terminated 3 days after the last control mosquito emerged. Leaves remained in the bowl for the duration of the assay. The median lethal doses (LD<sub>50</sub>s) were determined by probit analysis (SAS Institute, Inc., 1985).

**Age sensitivity study:** Mosquito larvae, age 2–7 days, were treated with *C. iria* leaves to determine which days were most sensitive. Mosquitoes (20 larvae per bowl, 4 replicates per time point), were hatched on 6 consecutive days and 500 mg of *C. iria* leaves (2 months old) was added when the youngest larvae were 2 days old. Leaves were added at the times noted and remained in the bowls for the duration of the assay. Adult emergence was measured as described above.

## RESULTS

To determine whether JH III within chopped leaves could disrupt development, a dose-response study was performed to assess adult emergence. Leaves were harvested from either 1- or 2-month-old plants and frozen until assayed. Figure 1 demonstrates that leaves from both ages caused significant larval mortality. The LD<sub>50</sub> of the 1-month-old leaves was 267 mg/100 ml H<sub>2</sub>O, whereas that of the 2-month-old leaves was 427 mg/100 ml H<sub>2</sub>O. Analysis of covariance indicated that dose and plant age were highly correlated ( $P < 0.0001$  and  $P < 0.0014$ , respectively). Leaves from the younger plant demonstrated a greater potency that correlates with RIA data showing that the younger plant contained significantly higher levels of JH III. The 1-month-old plant had 193 ± 5 µg JH III/g fresh leaf and the 2-month-old plant had 143 ± 9 µg JH III/g fresh leaf. In our analysis, 1-month-old *C. iria* had more JH III than mature plants. These data are in good agreement with titers previously reported by Toong et al. (1988) (Table 1).

Because JH III was suspected to be the active component suppressing adult development, a dose-response study comparing the effects of JH III purified from *C. iria* and the effects of frozen *C. iria* leaves was conducted. Using JH values calculated from the RIA, milligrams of leaf material were con-

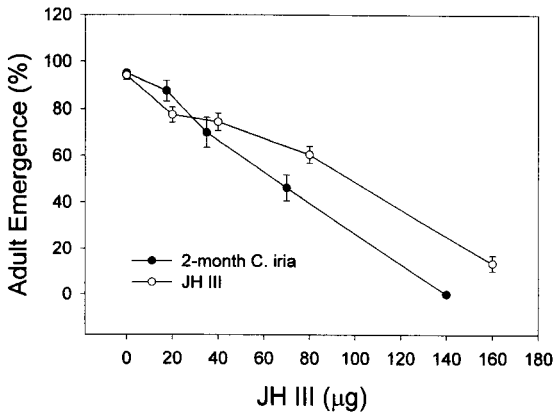


Fig 2. Effect of pure juvenile hormone III (JH III) and 2-month-old *Cyperus iria* on adult emergence of *Aedes aegypti*. The JH III purified from *C. iria* and 2-month-old leaves were added to 100 ml of water containing 4-day-old larvae. The JH III assays were tested at 8 bowls/treatment and *C. iria* at 4 bowls/treatment. When pupae appeared, the bowls were placed in individual cages. Successful emergence was determined by the mosquitoes' ability to fly from the bowl. Emergence was monitored for 3 days after the last control animal emerged. Controls consisted of bowls with either no plant material or 1,000 mg *Cyperus esculentus* (data not shown). All leaves used in this study were from frozen stock. Vertical bars represent SE. Absent SE bars indicate that SE was smaller than datum point.

verted to micrograms JH III equivalents. Figure 2 shows that highly purified JH III and equivalent JH in leaf material yielded a similar dose-response curve; however, the frozen plant material is more potent than pure JH III at higher doses.

Although the leaf-induced mortality appeared to mimic actions normally seen with highly purified JH, the presence of leaves might cause mortality by restricting larval feeding or respiration. To control for this possibility, *C. esculentus*, a closely related sedge containing no JH (Table 1), was tested. No mortality from *C. esculentus* was observed at the highest treatment level (1,000 mg). Larvae treated on day 4 with 1,000 mg of *C. esculentus* were measured 2 days later and compared with larvae receiving no leaves. No significant differences in body length (mouthparts to anal papillae) were observed (mean  $\pm$  SE mm for nontreated and *C. esculentus*-treated larvae were  $9.20 \pm 0.18$  and  $9.25 \pm 0.17$ , respectively). No significant difference was found in mean number of adults emerging per bowl for nontreated and *C. esculentus*-treated larvae ( $19.0 \pm 0.0$  and  $17.8 \pm 0.47$ , respectively). Thus, the possibility that leaves acted as a physical barrier to feeding or respiration can be eliminated and we can attribute larval mortality to JH leaching from the leaf.

In many insects the window of JH III sensitivity varies with larval age (Slama et al. 1974). Most studies on mosquito development focus on 3- to 4-

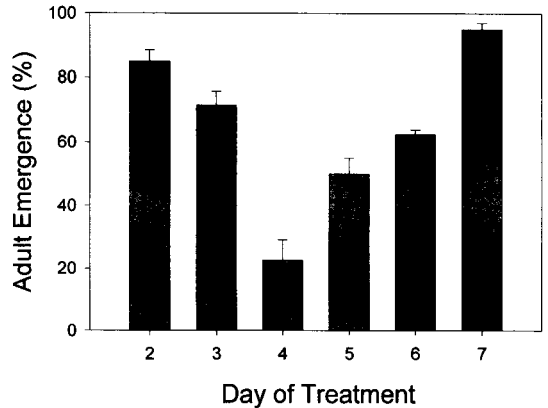


Fig 3. Effect of *Cyperus iria* leaf on larvae of different ages. Five hundred milligrams of *C. iria* leaves (from 2-month-old plants) was added to bowls containing 20 larvae and 100 ml of water. The larval ages at time of treatment were from 2 to 7 days. Day 7 contained a mixture of larvae and pupae. Successful emergence was determined by the mosquitoes' ability to fly from the bowl. Emergence was monitored for 3 days after the last control animal emerged. All leaves used in this study were from frozen stock. Each point represents the average of 4 replicates and vertical bars represent SE. Control treatments consisted of 1,000 mg of *Cyperus esculentus*.

day-old larvae (Henrick et al. 1975, Saxena et al. 1993, Perich et al. 1994). Figure 3 demonstrates that 4-day-old larvae are the most sensitive to *C. iria* leaves. Larvae treated on day 5 are more sensitive than those treated on day 6, followed by days 3 and 2. Samples treated on day 7 were a mixture of larvae and pupae that were insensitive to the *C. iria* leaves. No statistical difference between controls and insects treated on day 7 was observed. Thus, younger larvae are less susceptible to *C. iria* than are older larvae; however, as larvae approach pupation, developmental sensitivity to the leaves declines.

To ascertain if JH III is common to other *Cyperus* species, six species of *Cyperus* were assayed for JH III. The plants were chosen based upon availability and quarantine regulations. Because JH III levels were suspected to be age-related, only mature plants at seed formation were chosen for study. Table 1 shows that of the six *Cyperus* species assayed by RIA, only *C. iria* contains JH III.

## DISCUSSION

It is often difficult to attribute mortality to a single compound in plant material; however, in the case of *C. iria* the evidence clearly points to JH III. First, on a gram per gram basis, plants with higher JH levels were more lethal. Second, physical characteristics of the *C. iria*-treated larvae were congruent with treatment with highly purified JH III: larvae and pupae lacked melanization and adults were unable to escape the pupal exoskeleton (Bus-

vine 1978). Another classical result of excess JH, adult tarsi ensnared in the exoskeleton, was also clearly evident in treated insects (Spielman and Williams 1966). Finally, sublethal doses of *C. iria* increased the length of the life cycle by at least 1 day (data not shown).

Levels of JH III in *C. iria* varied with plant age. One-month-old plants contained 193  $\mu\text{g}$  JH III/g fresh leaf, whereas 2-month-old plants contained 143  $\mu\text{g}$  JH III/g fresh leaf. Toong et al. (1988) reported that 3-month-old mature plants, grown under greenhouse conditions, contained 151  $\mu\text{g}$  JH III/g fresh weight (Toong et al. 1988). Although younger plants have more JH, the optimal time to harvest *C. iria* for large-scale usage might be closer to 2 months because total plant mass would be maximal at this time. This trade-off must be considered when determining the optimal stage for harvest.

The biological relevance of JH III in *C. iria* remains unclear. Although the consumption of JH will eventually disrupt the development and reproduction of insects that feed on the plant, feeding periods would be prolonged, thus proving detrimental to the plant. One possible explanation is that JH III found in *C. iria* may serve a dual role, as an antiinsect/antigermination chemical. Extracts of *Cyperus serotinus* exhibited antiauxin and antigibberellin characteristics and inhibit rice germination (Komai et al. 1981). The extract consisted of farnesol and methyl farnesate, both precursors of JH III.

The state in which JH is delivered to the water has a significant effect on potency. Highly purified JH III is more potent than endogenous plant JH at doses lower than 30  $\mu\text{g}$  (Fig. 2). At doses greater than 30  $\mu\text{g}$ , endogenous plant JH III was more lethal. Plant material containing 140  $\mu\text{g}$  of JH III inhibited 99% of the adult emergence compared to 70% for 140  $\mu\text{g}$  pure JH III. The leaf may protect the hormone from water and direct light, factors that are instrumental in JH and JHA degradation (Henrick et al. 1975). Moreover, the leaf releases the hormone slowly, ensuring that a constant supply of hormone is present on the surface of the water for an extended period of time. This may explain why equivalent doses of purified JH III and unextracted JH III in leaf material display different  $\text{LD}_{50}\text{s}$ .

Three general levels of developmental disruption were observed depending upon the amount of leaf present in the bowl. The most severe condition, larval and pupal mortality, was caused by the highest dose of leaf. The cause of larval mortality was not examined but it was clear that high doses interfered with the insects' capability to melanize the exoskeleton, as the dead larvae and pupae displayed none of the typical dark cuticular markings (Busvine 1978). Intermediate doses of the leaf material yielded insects that survived larval-pupal transformation but failed to undergo adult eclosion. These insects began their emergence from the pupal exo-

skeleton but became ensnared in the old exoskeleton and were unable to escape. In contrast to insects exposed to high leaf material, these adults were normally pigmented and the exuvial fluid surrounding the pharate adult appeared extensively melanized upon adult emergence. It is possible that the exuvial fluid that normally aids in ecdysis becomes polymerized with melanin, entrapping the tarsi. Although high levels of JH normally inhibit melanization (Riddiford 1985), exposure to exceedingly high levels of JH during sensitive periods in the larval stage may have permanently shut off JH synthesis. Thus, in the pupal stage, the lack of endogenous or exogenous JH may lead to the high levels of observed melanization (Schwartz and Goodman, unpublished observation). That exogenous JH or analogs can block JH synthesis permanently has been observed in other insects (Baker et al. 1986). Larvae treated with low doses of leaf material showed none of the above responses and appeared normal; however, physiologic and biochemical abnormalities may have been present. These insects developed slower than controls, taking an extra day to complete their egg to adult development (data not shown).

Although *C. iria* is distributed throughout the tropical regions of the world and could be available for use in regions where mosquito-borne diseases are endemic, other species in the same family (in which there are >3,600 species [Huxley et al. 1992]), may actually contain more JH III. Indeed, Toong (personal communication) has suggested at least one other species, *Cyperus aromaticus*, may contain even higher levels of JH III than does *C. iria*. Komai et al. (1981) reported that another species of *Cyperus*, *C. serotinus*, contains the precursors for JH III but no further studies were conducted to determine if the plants contained JH. We tested the JH content of several different species of *Cyperus* and found that none contained detectable levels of JH. These included species native to southeast or western Asia (*C. esculentus*), Africa (*C. albobstriatus*, *C. alternifolius*, and *C. papyrus*), and South America (*C. miliifolius*) (Bailey 1976). Based on this preliminary survey, the most readily available species of *Cyperus* do not contain JH III. However, given the enormous number of species in this family, it is likely that other species may also contain significant levels of JH III.

Henrick et al. (1975) reported the  $\text{LD}_{50}$  for methoprene to be 0.00017 ppm, whereas our data indicate that the  $\text{LD}_{50}$  for *C. iria* is approximately 5 times greater. Although the  $\text{LD}_{50}$  for *C. iria* makes the plant less effective than the commercially available JHAs, the ubiquitous presence of the plant in endemic areas and its relative cost make extensive field studies attractive. Moreover, the low mammalian toxicity of the JHs makes it possible to use the plant in water used for human consumption.

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