

ESTABLISHMENT OF *LIPOLEXIS OREGMAE*
(HYMENOPTERA: APHIDIIDAE) IN A CLASSICAL BIOLOGICAL
CONTROL PROGRAM DIRECTED AGAINST THE BROWN CITRUS APHID
(HOMOPTERA: APHIDIDAE) IN FLORIDA

ANAND B. PERSAD^{1*}, MARJORIE A. HOY¹ AND RU NGUYEN²

¹Department of Entomology and Nematology, University of Florida, Gainesville, FL 32611-0620

*Current Address: Davey Institute, P.O. Box 5193, Kent OH 44240-5193

²Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Gainesville, FL

ABSTRACT

The parasitoid *Lipolexis oregmae* Gahan (introduced as *L. scutellaris* Mackauer) was imported from Guam, evaluated in quarantine, mass reared, and released into citrus groves in Florida in a classical biological control program directed against the brown citrus aphid, *Toxoptera citricida* Kirkaldy. Releases of 20,200, 12,100, and 1,260 adults of *L. oregmae* were made throughout Florida during 2000, 2001, and 2002, respectively. To determine if *L. oregmae* had successfully established, surveys were conducted throughout the state beginning in the summer of 2001 and continuing through the summer of 2003. Parasitism during 2001 and 2002 was evaluated by holding brown citrus aphids in the laboratory until parasitoid adults emerged. *Lipolexis oregmae* was found in 10 sites in 7 counties and 4 sites in 3 counties with parasitism rates ranging from 0.7 to 3.3% in 2001 and 2002, respectively. Laboratory tests indicated that high rates of mortality occurred if field-collected parasitized aphids were held in plastic bags, so a molecular assay was used that allowed immature *L. oregmae* to be detected within aphid hosts immediately after collection. The molecular assay was used in 2003 with the brown citrus aphids and with other aphid species collected from citrus, weeds, and vegetables near former release sites; immatures of *L. oregmae* were detected in black citrus aphids, cowpea aphids, spirea aphids, and melon aphids, as well as in the brown citrus aphid, in 4 of 8 counties sampled, with parasitism ranging from 2.0 to 12.9%, indicating that *L. oregmae* is established and widely distributed. Samples taken in Polk County during Oct 2005 indicated that *L. oregmae* has persisted. The ability of *L. oregmae* to parasitize other aphid species on citrus, and aphids on other host plants, enhances the ability of *L. oregmae* to persist when brown citrus aphid populations are low.

Key Words: *Lipolexis oregmae*, classical biological control, *Toxoptera citricida*, citrus, *Lysiphlebus testaceipes*, establishment, alternative hosts, Florida

RESUMEN

El parasitoide *Lipolexis oregmae* Gahan (introducido como *L. scutellaris* Mackauer) fue importado de Guam, evaluado en cuarentena, criado en masa y liberado en huertos de cítricos en un programa de control biológico clásico dirigido contra el áfido pardo de cítricos, *Toxoptera citricida* Kirkaldy. Se hicieron liberaciones de 20,200, 12,100, y 1,260 adultos de *L. oregmae* a través de la Florida durante los años de 2000, 2001, y 2002, respectivamente. Para determinar si *L. oregmae* ha logrado en establecer, se realizaron sondeos a través del estado empezando en el verano del 2001 y continuando hasta el final del verano del 2003. El parasitismo durante 2001 y 2002 fue evaluado con el mantenimiento de individuos del áfido pardo de los cítricos en el laboratorio hasta que los adultos emergieron. *Lipolexis oregmae* fue encontrado en 10 sitios en 7 condados y con tasas de parasitismo en 4 sitios en 3 condados entre 0.7 a 3.3% en el 2001 y 2002, respectivamente. Las pruebas del laboratorio indicaron que las tasas altas de mortalidad fueron posibles si los áfidos con parasitos recolectados en el campo fueron mantenidos en bolsas plásticas, entonces un ensayo molecular fue usado con lo que permitió la detección de inmaduros de *L. oregmae* dentro de los hospederos de áfidos inmediatamente después de la recolección. El ensayo molecular fue usado en el 2003 con individuos del áfido pardo de los cítricos y con otras especies de áfidos recolectados sobre cítricos, malezas y hortalizas cerca de los sitios donde los parasitoides fueron liberados anteriormente; inmaduros de *L. oregmae* fueron detectados en individuos del áfido negro de los cítricos, el áfido del caupí, el áfido spirea y el áfido del melón, además del áfido pardo de los cítricos en 4 de los 8 condados muestreados, con la tasa del parasitismo entre 2.0 a 12.9%, indicando que *L. oregmae* estaba establecido y ampliamente distribuido. Las muestras tomadas en el Condado de Polk durante octubre del 2005 indicaron que *L. oregmae* ha persistido.

La capacidad de *L. oregmae* para parasitar otras especies de áfidos sobre cítricos y otros áfidos sobre otras plantas hospederas, incrementa la capacidad de *L. oregmae* para persistir cuando las poblaciones del áfido pardo de los cítricos están bajas.

The brown citrus aphid, *Toxoptera citricida* Kirkaldy (Homoptera: Aphididae), was first detected in Florida in Nov 1995 in Dade and Broward counties and rapidly became established on the 860,000 acres (348,031 ha) of citrus then grown throughout the state (Halbert & Brown 1996; Fasulo & Halbert 2001). The brown citrus aphid originated in Asia but invaded citrus-growing areas in the Caribbean, as well as Central and South America prior to invading Florida (Stoetzel 1994; Rocha-Pena et al. 1995; Yokomi & Tang 1996). The brown citrus aphid causes economic losses in both groves and citrus nurseries (Kozaki 1994; Knapp et al. 1996; Tsai 1998). Adults and nymphs deplete the sap of young citrus foliage (flush), and high populations can destroy the growing tips of citrus flush and produce copious amounts of honeydew, which allows sooty mold to grow. More importantly, this aphid is an efficient vector of citrus tristeza virus (CTV) (Kozaki 1994; Yokomi et al. 1993, 1994; Rocha-Pena et al. 1995). Citrus propagated on sour orange rootstock is especially susceptible to CTV and approximately 14% of orange trees (12 million) and 42% of grapefruit trees (about 5.3 million) trees on sour orange rootstock in Florida were vulnerable to CTV after the invasion of the brown citrus aphid (Brown & Stover 2002).

Native natural enemies of the brown citrus aphid in Florida include predators, fungal pathogens, and a parasitoid (Evans & Stange 1997; Michaud 1998; Fasulo & Halbert 2001). *Lysiphlebus testaceipes* Cresson attacks the brown citrus aphid in Florida, but was considered ineffective because it appeared to complete its development only rarely due to a phenomenon called incomplete parasitism, which results in a failure of adults to emerge from the mummy (Carver 1984; Stary 1989; Yokomi & Tang 1996; Michaud & Browning 1999). Four species of hyperparasitoids parasitize *L. testaceipes* larvae within the brown citrus aphid in Florida (Evans & Stange 1997), which could limit its effectiveness. Thus, the parasitoid component of natural enemies attacking the brown citrus aphid in Florida was limited and the addition of another parasitoid species to the natural enemy guild was expected to improve suppression of brown citrus aphid populations (Yokomi et al. 1993; Hoy & Nguyen 2000).

Unfortunately, relatively few parasitoids of the brown citrus aphid have been identified (Carver 1978, 1984; Yokomi et al. 1993; Michaud 1998). *Lysiphlebia japonica* (Ashmead) was found associated with the brown citrus aphid in Japan, was imported into Florida and Puerto Rico in 1996 and released, but there is no evidence it has es-

tablished in Florida (Evans & Stange 1997; Deng & Tsai 1998). *Lysiphlebia mirzai* Shufa-Uddin was imported into Florida from China in 1996, but was not released (R. Nguyen, Personal Communication). *Aphidius colemani* Viereck was imported from Chile, but also was not released in Florida (R. Nguyen, Personal Communication). *Aphelinus gossypii* Timberlake was introduced into Florida from Hong Kong in 1962 for the control of spirea aphid, *Aphis spiraeicola* Patch, but has been recovered only rarely from the brown citrus aphid (Evans & Stange 1997).

Lipolexis scutellaris Mackauer (Hymenoptera: Aphidiidae) was reported to be a koinobiont parasitoid of the brown citrus aphid, as well as of the black citrus aphid, *Toxoptera aurantii* (Boyer de Fonscolombe), the cowpea aphid, *Aphis craccivora* Koch, and the cotton or melon aphid, *Aphis gossypii* Glover, on citrus and vegetables in India, Pakistan, southern China, Taiwan, and Japan (Dharmadhikari & Ramaseshiah 1970; Stary & Zeleny 1983; Singh & Pandey 1997; Singh et al. 2000). Information obtained on the biology of *L. scutellaris* in India indicated this parasitoid primarily attacks second- and third-instar aphids, that females show a high degree of discrimination, and diapause is exhibited in the mummy stage (Shuja-Uddin 1977; Radhakrishnan & Muraliedharan 1992; Singh et al. 2000).

Lipolexis scutellaris was reported by P. Stary and R. Miller (personal communications) to be an accidental immigrant to Guam, possibly from the Philippines. In a personal communication, R. Miller indicated that *L. scutellaris* "... has established itself as the major aphidiid parasitoid of crop aphids on Guam. Its host range on Guam consists of *Toxoptera citricida*, *Aphis gossypii*, *Aphis craccivora* and *Aphis spiraeicola*. It is most commonly found, and found in the greatest densities on *T. citricida* on tangerine, lemon, calamondin, and orange". The fact that it was most commonly found on brown citrus aphid in Guam suggested it could be an important natural enemy of the pest in Florida, should it be established. Because Stary (1988) stated that genetic variability within species of aphidiids is substantial, even suggesting that some species may consist of species complexes, it is not clear whether the Indian and Guam populations of *L. scutellaris* are equivalent in their developmental rate, host species preferences, adaptation to climatic conditions, or other important biological traits. As a result, the population of *L. scutellaris* we worked with is called the Guam biotype.

After the Guam biotype was introduced into Florida, *L. scutellaris* was found to be a synonym

of *L. oregmae* Gahan by Miller et al. (2002). However, research conducted on the Guam biotype was published under *L. scutellaris* (Hoy & Nguyen 2000; Hill & Hoy 2003; Persad & Hoy 2003). Walker & Hoy (2003) showed that *L. oregmae* can oviposit and develop in all 4 instars of the brown citrus aphid in Florida and that it has a very high reproductive rate on this host. In laboratory trials, females reared from second instars produced a mean of 100 progeny, while the larger females reared from fourth-instar hosts produced an average of 205 progeny. Thus, *L. oregmae* has the potential to be a successful natural enemy of the brown citrus aphid in Florida, having a low incidence of incomplete parasitization and a high reproductive rate on the target pest.

We report on the releases of *L. oregmae* into citrus groves in Florida between 2000 and 2002 and the field evaluations conducted during 2001 through 2003 to evaluate establishment, overwintering, and spread. During 2001 and 2002, establishment and overwintering was confirmed by collecting citrus flushes infested with brown citrus aphids and holding the aphids in the laboratory until adult parasitoids emerged. In the surveys conducted during 2003, aphids found in citrus groves and in nearby weeds, ornamentals, or vegetables within 3 miles (4.8 km) of release sites were evaluated for the presence of *L. oregmae* with a High-fidelity PCR protocol.

MATERIALS AND METHODS

Importation of *L. oregmae*

Adults of *L. oregmae* were shipped from Guam into the high security quarantine facility at the Division of Plant Industry Department of Agriculture and Consumer Services, in Gainesville, Florida under USDA-APHIS Permit 954945 on Aug 19, 1999. On Dec 6, 1999 a subculture was transferred to the Department of Entomology and Nematology quarantine facility at the University of Florida, Gainesville with a permit.

Mass Rearing of *L. oregmae*

Lipolexis oregmae was cultured in a synchronous tritrophic rearing system (Hill & Hoy 2003; Walker & Hoy 2003). Potted citrus plants were infested with mixed stages of brown citrus aphids and then exposed to parasitoids within plastic cylinders in the Division of Plant Industry quarantine facility or in mesh cages in the quarantine laboratory of the Department of Entomology and Nematology, University of Florida, Gainesville. Parasitoids emerging from the rearing cages were stored in plastic vials (6.5 × 2.5 cm) containing a fluted paper (2 × 5 cm) for refuge and a moistened honey-saturated absorbent paper strip as a nutrient source. Parasitoids were stored in groups of

up to 50 individuals per vial and were either used to initiate cultures or shipped overnight to collaborators in styrofoam boxes cooled with frozen cold packs or taken directly to citrus groves within 1-3 d of emergence for release.

Release Protocol for *L. oregmae*

All releases were made in citrus groves with abundant flush and had at least 3 to 5 trees well infested with brown citrus aphids (>3,000 aphids each). The location of each release site was recorded with a handheld Geographic Positioning Systems receiver (Magellan GPS 310, Orbital Products, San Dimas, CA.) as well as on a Florida state map (Florida Atlas and Gazetteer 1997, DeLorme, Maine). During releases, the cap on the vial containing parasitoids was unscrewed and the vial held upright under the brown citrus aphid colonies. Most release sites received single releases of 100 to 200 newly emerged *L. oregmae* adults, although some sites received multiple releases. Females were often observed stinging brown citrus aphids moments after their release. No releases were carried out in heavy rain or at locations where irrigation sprays would have washed the aphids off the foliage, or in groves where pesticides were recently applied.

Effect of Storing on Fecundity of Released *L. oregmae* Females

To confirm that we could store females for 1-3 d at 15°C without a loss in vigor when it was difficult to release parasitoids on the day of emergence, 10 females of *L. oregmae* were individually placed on a potted citrus plants infested with 250-300 aphids of mixed stages on the day of parasitoid emergence and left for 24 h. The plant was covered with a ventilated plexiglass cylinder and a moist cotton pad and honey-saturated paper strip within for water and nutrients, respectively. Female parasitoids were allowed to mate individually with 1-d-old males in gelatin capsules (size 00) and then exposed to 5 aphids for 5 min prior to introducing them to the aphid-infested plants. Any female that did not mate or did not oviposit into aphids was not used.

After female parasitoids were removed, the plants were held in the laboratory at 22-24°C, 55-65% RH and 16L:8D cycle until emergence of adult parasitoids 13-14 d later. Any replicate in which the female could not be located after 24 h was rejected. The experiment was repeated with 10 females that were stored in a growth chamber at 15°C under a 16L:8D cycle for 3 d and 10 females stored for 6 d. All emerging parasitoids from the 10 replicates of the 3 treatments were counted and One-way Analysis of Variance and Least Significant Difference (Statview ver 5.0) was used to compare the mean fecundity for each treatment.

Survey Sites and Sampling Protocol for 2001 and 2002

Survey sites during 2001 and 2002 were all former release sites or within a 4.8-km (3-mile) radius of former release sites. All flushes on all trees that could be examined within 45 min were inspected, and all flushes infested with brown citrus aphids were collected. Citrus flushes were placed between loosely folded paper towels in a 'Ziploc' plastic bag (Fisher Scientific) (22 × 14 cm) which was moderately inflated and stored in an insulated cooler (65 × 54 × 24 cm). The temperature (10-17°C) within the cooler was maintained by a single layer of 'blue ice' packets and separated from direct contact with the sample bags by 4 layers of heavy plastic sheeting. Once in the laboratory, bags were wiped inside to remove condensation and were fully inflated with a laboratory air pump, resealed, and stored on bench tops under a 16L:8D photoperiod.

The samples were checked daily at 900, 1200, and 1600 h for adult parasitoid emergence; paper towels within each bag were replaced if wet. Because most mummified brown citrus aphids containing *L. oregmae* form in or on the soil (Hill & Hoy 2003), freshly collected flush samples rarely contained mummies. Adult wasps were identified with the guidelines of Evans & Stange (1997). Samples were held for 8 to 10 d, by which time any brown citrus aphids containing eggs or larvae of either parasitoid species would become mummified.

Mortality Associated with Sampling Protocol

To determine whether the sampling protocol caused significant mortality of aphids or immature parasitoids, a laboratory assessment was conducted. Each of 10 third-instar brown citrus aphids was exposed to either *L. oregmae* or *L. testaceipes* in a petri dish arena according to methods described by Persad & Hoy (2003), and the parasitized aphids were returned to tender new leaves on potted citrus. To confirm parasitism, 3 d later a third of the exposed aphids were removed and dissected in 0.7% saline under a microscope to record the number of parasitoid eggs or larvae. One-third of the aphids were removed along with leaves and stored in bags as described above, and the remaining third were reared on the flushed citrus plants. If there was less than the expected 97 to 100% parasitism rate in any group (Persad & Hoy 2003), that trial was rejected. Because up to 75% mortality occurred before adult parasitoids emerged in both the plastic bags and the rearing treatments, a molecular test was developed (Persad et al. 2004) and used during 2003 to monitor field-collected foliage containing aphids for the presence of *L. oregmae*.

High-fidelity PCR Protocol for Monitoring *L. oregmae* and *L. testaceipes* in 2003

In 2003, field samples were split into 2 treatments; 1 consisted of pooled (potentially parasitized) aphids (70% of the sample obtained from a particular location), and the other consisted of individual parasitoid larvae dissected from the remaining 30% of the aphids. The assay conducted on pooled aphids (36 aphids or fewer) used the High-fidelity PCR protocol developed by Persad et al. (2004). For the pooled aphids, DNA was extracted from groups of up to 36 aphids (and potential parasitoids) in 0.5-ml thick-walled eppendorf tubes, each containing 50 µl of 5% Chelex (Bio-Rad, Hercules, CA) resin. The extraction took 1 h at 60°C and 5 min at 94°C (Edwards & Hoy 1993). To grind the aphids and parasitoids in Chelex, a pestle was made by slowly heating a standard pipette tip which was then inserted into an empty 0.5-mL eppendorf tube so that the tip assumed the shape of the base of the tube to form a close-fitting pestle, which was used once only. The remaining aphids (30%) were dissected in saline under a microscope and any parasitoid larva found was individually identified by High-fidelity PCR protocol because larvae of *L. oregmae* and *L. testaceipes* cannot be discriminated after the first instar.

One microliter of each Chelex preparation was used for High-fidelity PCR, which was performed in a 50-µL reaction volume containing 50 mM Tris, pH 9.2, 16 mM ammonium sulfate, 1.75 mM MgCl₂, 350-µM each of dATP, dGTP, dCTP, dTTP, 800 pmol of primers, 1 unit *Tgo* DNA polymerase, and 5 units of *Taq* DNA polymerase (Roche Molecular Biochemicals). The PCR primers used code for the nuclear rRNA ITS-2 region and include an *L. oregmae*-specific forward primer (LO-ITSF 5'-GGCCAGTTGTCGAGTCC-3') based on the complete ITS-2 sequence. DNA from any larva not identified as *L. oregmae* was retested with a species-specific primer to determine the presence of *L. testaceipes*, the only other primary parasitoid recovered during the surveys in 2001 and 2002. The *L. testaceipes*-specific forward primer (LT-ITSF 5'-CTAGCGATAAATGAATGTTTC-3') was designed after obtaining a partial ITS-2 sequence from *L. testaceipes* (Persad et al. 2004). PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and photographed under UV light. The *L. testaceipes*-specific primers produced 520-bp bands, while those of *L. oregmae* produced 270-bp bands.

RESULTS AND DISCUSSION

Importation and Quarantine Evaluation

The application to release *L. oregmae* was reviewed by the Institute of Food and Agricultural Sciences of the University of Florida and the

Division of Plant Industry of the Florida Department of Agriculture and Consumer Services. The identity of *L. oregmae* was confirmed by Peter Stary, Institute of Entomology, Academy of Sciences, Czech Republic. Specimens of *L. oregmae* were deposited at the Florida State Collection of Arthropods, Division of Plant Industry (DPI), Gainesville, as voucher specimens FBCL-2002-2. The parasitoid was released under DPI-Florida Department of Agriculture and Consumer Services permit number 208-00-026, dated 18 February 2000. A copy of the application to release is available at http://ipm.ifas.ufl.edu/reports/L_scutellaris_5.31.00.pdf, where a description is given of the potential hosts of *L. oregmae* in Florida and its potential nontarget effects. The potential benefits of establishing *L. oregmae* in Florida's citrus were considered to be greater than potential risks to native aphid species because there are no known threatened or endangered aphids in Florida (S. Halbert, personal communication).

Releases of *L. oregmae*

After approval of the application to release *L. oregmae* was obtained on 21 Jun 2000, releases were made in 15 counties throughout the approximately 348,031 ha of citrus in Florida (Table 1). Releases of approximately 20,200, 12,100, and 1,260 adults of *L. oregmae* were made in citrus groves in Florida during 2000, 2001, and 2002, respectively.

The 24-h test of fecundity of *L. oregmae* females conducted to confirm that the holding and shipping conditions were suitable was not different for females held for 1 or 3 d at 15°C. A mean (\pm SE) of 28.6 (\pm 2.1) adult parasitoid progeny were produced if their mothers were held for 1 d, and a mean of 39.4 (2.9) progeny were produced if females were first held for 3 d at 15°C. However, significantly fewer progeny (22.6, SE = 2.1) were produced if the mothers were held for 6 d at 15°C (One-way ANOVA, $F = 2.81$, $df = 29$, $P = 0.02$). Because most parasitoids were shipped on the day of emergence and were released by collaborators the next day or releases were made by the authors within 3 d of adult parasitoid emergence, females should have been sufficiently fit that they could locate hosts and begin oviposition after release. Observations made by the authors of females immediately after release in the field indicated that females could find and attack hosts readily.

Recovery of *L. oregmae* from Citrus Groves in Florida in 2001 and 2002

Recoveries of *L. oregmae* at or near release sites occurred in 10 citrus groves in 4 counties during 2001 and in 4 groves in 3 counties in 2002 (Table 1). Between Mar and Oct, there are periods (typically 3 or 4) when citrus trees are putting out

large amounts of new growth (flush) suitable for the development of large brown citrus aphid populations; during the rest of the growing season, aphid populations are often very low due to a lack of tender new flush. Although monitoring of citrus groves took place between Mar and Oct each year, the majority of brown citrus aphids were found between Jun and Aug. The percentage parasitism of brown citrus aphids by *L. oregmae* at these sites ranged from 0.7 to 3.3%. Because brown citrus aphids containing *L. oregmae* pupate in or on the soil, predation by ground foragers such as red imported fire ants, *Solenopsis invicta* Buren, may contribute to the low incidence of *L. oregmae* in these samples (Hill & Hoy 2003; Persad & Hoy 2004). For unknown reasons, the number of groves found to contain brown citrus aphids decreased from 58 sites out of 122 (48%) monitored in 2001 to 12 of 70 (17%) evaluated in 2002. This made evaluation of the establishment and dispersal of *L. oregmae* difficult, so we decided to evaluate other aphid species on additional host plants in 2003 to resolve whether *L. oregmae* was established.

Recovery of *L. oregmae* within 5 Aphid Species in or near Citrus Groves in Florida in 2003 Detected by High-fidelity PCR

High-fidelity PCR assays detected the presence of *L. oregmae* in 5 species of aphids (*Aphis spiraeicola*, *A. gossypii*, *A. craccivora*, *T. citricida*, and *T. aurantii*) collected from citrus foliage (c), vegetables (v), or weeds (w) in 4 of the 8 counties surveyed in Florida during 2003 (Table 2). The percentage parasitism of these aphids by *L. oregmae* ranged from 2.0 to 12.9% when parasitism was estimated from larvae dissected out of their aphid hosts (Table 2). Of the 7 aphid species collected in the survey, only *Myzus persicae* (v) and *Uroleucon pseudambrosiae* (w) did not contain immature *L. oregmae*.

Lipolexis oregmae was found in both the individual and group analyses of aphids collected from Alachua, Indian River, or Lake counties (Table 2). In Hendry county, assays on individually dissected parasitoid larvae were negative for *L. oregmae*, but pooled aphid samples from that county yielded two groups positive for *L. oregmae*. The absence of *L. oregmae* larvae in the dissected aphids suggests that parasitism was very low in this site, but the positive PCR results from the pooled samples from the same location indicate establishment at low levels by *L. oregmae*. Sensitivity analysis has shown the High-fidelity PCR assay can detect a single parasitized aphid when combined with up to 499 unparasitized aphids (Persad et al. 2004).

An advantage of the High-fidelity PCR assay is that it is sensitive and allows monitoring of large numbers of pooled aphids for the presence of

TABLE 1. NUMBER OF *LIPOLEXIS OREGMAE* (LO) RELEASED IN EACH COUNTY BETWEEN 2000 AND 2002, NUMBER OF SITES SAMPLED FOR *TOXOPTERA CITRICIDA* (TC), NUMBER OF SITES POSITIVE FOR LO, AND PERCENTAGE PARASITISM IN A SURVEY OF 15 COUNTIES IN FLORIDA DURING 2001 AND 2002.

County sampled	No. Lo released			No. sites sampled		No. sites with Tc		No sites with Lo and % parasitism			
	2000	2001	2002	2001	2002	2001	2002	2001	% par.	2002	% par.
Alachua	1500	1650	350	6	6	3	1	2	1.2	1	1.0
Collier	750	300	—	7	¹ NS	2	NS	NS	—	NS	—
Dade	300	50	—	NS	NS	NS	NS	NS	—	NS	—
Desoto	400	—	—	9	NS	3	NS	NS	—	NS	—
Hardee	750	300	—	4	1	1	0	0	—	0	—
Hendry	3000	2600	—	10	6	4	2	0	—	0	—
Hernando	400	450	—	4	4	4	0	2	0.8	0	—
Highland	350	—	—	4	NS	4	NS	NS	—	NS	—
Indian River	3500	1900	120	9	5	5	3	1	3.3	1	2.6
Lake	1750	1450	450	23	11	11	0	2	1.3	0	—
Orange	1050	600	—	7	7	2	0	0	—	0	—
Palm Beach	600	200	—	3	4	3	2	1	0.7	0	—
Pasco	700	350	—	7	5	4	0	1	1.1	0	—
Polk	1950	850	340	24	12	9	1	0	—	0	—
St. Lucie	3200	1300	—	5	9	3	3	1	1.5	2	—
Mean ± SE	—	—	—	—	—	—	—	—	1.4 ± 0.3	—	1.8 ± 0.8
Total	20,200	12,100	1260	122	70	58	12	10	—	4	—

¹NS= site not sampled; all sites were either release sites or were <4.8 km from former release sites. All sites where *L. oregmae* was found in 2001 were sampled in 2002.

TABLE 2. DETECTION OF *LIPOLEXIS OREGMAE* (LO) AND *LYSIPHLEBUS TESTACEIPES* (LT) OBTAINED FROM APHIDS COLLECTED FROM CITRUS (C), VEGETABLES (V) OR WEEDS (W) WITH HIGH-FIDELITY PCR PROTOCOL AND SPECIES-SPECIFIC PRIMERS ON PARASITOID LARVAE OBTAINED BY DISSECTION (30% OF SAMPLE) OR ON POOLED GROUPS OF ≤36 INTACT APHIDS (70% OF SAMPLE) FROM 8 COUNTIES IN FLORIDA DURING 2003.

County	Aphid species (from citrus, vegetables or weeds)	No aphids collected (from c, v, or w)	No larvae dissected from 30% of sample	No. larvae positive by PCR for		% dissected aphids parasitized by Lo	No. groups tested by PCR	No. groups positive for Lo
				Lo	Lt			
Alachua	<i>Aphis spiraecola</i> (w)	122	5	2	3	5.5	2	2
	<i>A. gossypii</i> (c, v)	139 (9, 130)	13 (0,13)	1	12	2.0	3	3
	<i>A. craccivora</i> (v, w)	55 (4, 51)	8 (1, 7)	0	8	—	1	1
	<i>Toxoptera citricida</i> (c)	361	17	5	12	4.6	4	2
Broward	<i>A. spiraecola</i> (c, v, w)	23 (5, 11, 7)	0	0	0	—	1	0
	<i>Myzus persicae</i> (v)	178	22	0	22	—	3	0
	<i>T. citricida</i> (c)	411	9	0	9	—	5	0
Hendry	<i>A. gossypii</i> (v)	127	3	0	3	—	2	2
	<i>T. citricida</i> (c)	67	14	0	14	—	1	1
	<i>T. aurantii</i> (c)	14	0	—	—	—	1	0
Indian River	<i>A. spiraecola</i> (c, w)	343 (0, 343)	8	5	3	4.9	5	5
	<i>A. gossypii</i> (c, w)	104 (0, 104)	13 (0, 13)	4	9	12.9	2	1
	<i>Uroleucon pseudambrosiae</i> (w)	54	0	—	—	—	5	0
	<i>T. citricida</i> (c)	22	2	0	2	—	1	0
Lake	<i>A. gossypii</i> (c, v)	77 (12, 55)	6 (0, 6)	0	6	—	2	1
	<i>T. citricida</i> (c)	118	24	2	22	5.7	2	2
Orange	<i>A. gossypii</i> (c)	34	0	0	0	—	1	1
	<i>T. citricida</i> (c)	106	12	1	11	2.9	3	2
Palm Beach	<i>A. spiraecola</i> (v)	41	0	—	—	—	1	0
	<i>T. citricida</i> (c)	164	4	0	4	—	3	0
	<i>T. aurantii</i> (c)	14	0	—	—	—	1	0
Polk	<i>A. craccivora</i> (v, w)	35 (17, 18)	0	—	—	—	1	0
	<i>T. citricida</i> (c)	11	0	—	—	—	1	0
Mean % ± SE	—	—	—	—	—	5.5 ± 1.3	—	—

L. oregmae (as well as of *L. testaceipes*). Furthermore, the PCR assay eliminates losses from mortality of immature parasitoids while they are held in plastic bags until adult parasitoids emerge. The High-fidelity PCR assay takes less time than rearing the parasitoids in bags because *L. oregmae* can be detected within 1 d after the sample is collected. The disadvantage of conducting the PCR assay on pooled aphids is that it provides only qualitative data on the presence of *L. oregmae* and it is expensive. However, the High-fidelity PCR assay allows us to evaluate larger numbers of aphids and sites when monitoring for establishment and distribution.

Lysiphlebus testaceipes and Hyperparasitoids

Lysiphlebus testaceipes was collected in all brown citrus aphid samples in which *L. oregmae* was found during the 3 field seasons. This suggests that *L. testaceipes* may be a better parasitoid of the brown citrus aphid than previously thought. For example, of 158 parasitoid larvae dissected from 5 aphid species during 2003 and assayed by High-fidelity PCR, 140 (89%) were *L. testaceipes* (Table 2). Several authors (Michaud 1998; Michaud & Browning 1999; Yokomi & Tang 1996) have rated *L. testaceipes* as ineffective because few adults emerge from brown citrus aphid mummies located on foliage due to the phenomenon called incomplete parasitization. However, Persad & Hoy (2003) discovered that a large proportion of brown citrus aphids parasitized by *L. testaceipes* mummify off the citrus foliage and a high proportion of these mummies can produce adults. We conclude that *L. testaceipes* is an effective parasitoid of the brown citrus aphid in citrus groves in Florida.

Two species of hyperparasitoids were recovered from the field-collected foliage held in plastic bags in 2001 and 2002. These comprised approximately 1.1 and 2.1% of all emerged parasitoids, respectively. They were identified as *Pachyneuron aphidis* Bouche and the '*Alloxysta megourae* complex'. Because the bags contained adults of *L. testaceipes*, as well as *L. oregmae*, we could not resolve which parasitoid produced these hyperparasitoids. To resolve whether these hyperparasitoids could develop on *L. oregmae*, we placed potted citrus trees containing brown citrus aphids parasitized by *L. oregmae* in the citrus grove located next to the Department of Entomology and Nematology building in Gainesville during Jun 2002; both hyperparasitoids were recovered at low rates (1-2%), confirming that these two hyperparasitoids will attack *L. oregmae*.

The results of surveys conducted in 15 counties containing approximately 348,031 ha of citrus between 2001 and 2003 show that *L. oregmae* was recovered from multiple sites some distance from the original release sites. Because *L. oregmae* was

not released after 2002, yet was found in multiple sites during 2003, *L. oregmae* appears to have successfully persisted, overwintered, and dispersed from the original release sites in Florida citrus groves.

Because brown citrus aphids collected from Polk County during Oct 2005 produced adults of *L. oregmae*, this parasitoid has persisted in Florida since 2002. Interestingly, *L. oregmae* was discovered during 2004 to be well established on brown citrus aphids in Jamaica, where it was fortuitously introduced from an unknown source (Hoy et al. unpublished data). It is likely that *L. oregmae* was introduced into Jamaica from Florida because Florida is the only known location where it has been released and established in the Western Hemisphere. Because *L. oregmae* parasitizes several aphid hosts, it could have been introduced on aphid species and host plants other than citrus.

During the 2001 through 2003 surveys in Florida, *L. testaceipes* was found consistently and was the only other primary parasitoid of brown citrus aphid. Its abundance raised the question as to whether *L. testaceipes* would interfere with the establishment of, or abundance of, *L. oregmae*. However, interspecific competition studies indicated that neither *L. oregmae* nor *L. testaceipes* were intrinsically superior to the other when reared on the brown citrus aphid in the laboratory (Persad & Hoy 2003).

Brown citrus aphids parasitized by *L. oregmae* do not mummify on the foliage, as is common with many other parasitized aphids. These mummies can be preyed on by the red imported fire ant, *Solenopsis invicta* Buren, and the Asian cockroach, *Blattella asahinai* Mizukubo (Hill & Hoy 2003; Persad & Hoy 2004). Furthermore, red imported fire ants forage in citrus trees and will selectively remove brown citrus aphids parasitized by *L. oregmae* (Persad & Hoy 2004). Because red imported fire ants are very abundant in citrus groves throughout Florida, their predation on parasitized aphids or mummies may be reducing the effectiveness of *L. oregmae* as a natural enemy of the brown citrus aphid in Florida.

Our data indicate that sampling methods designed to evaluate parasitism of brown citrus aphids by either *L. oregmae* and *L. testaceipes* should take into account the fact that the majority of parasitized brown citrus aphids move off the foliage to mummify. As a result, mortality estimates based on the few mummies retained on citrus foliage will underestimate mortality. Our data also indicate that *L. oregmae* and *L. testaceipes* are coexisting in citrus groves in Florida.

The use of alternative aphid host species on citrus, vegetables, and weeds was expected, and the survey conducted during 2003 confirms that *L. oregmae* is using these alternative hosts in Florida. *Lipolexis oregmae* is oligophagous (Stary

1988; Stary & Ghosh 1983; Stary & Zeleny 1983; Ahmad & Singh 1996) and all aphid species that we found parasitized are considered pests of economic importance. The use of alternative aphid species may prove advantageous for the efficacy of *L. oregmae* in citrus in Florida because it could allow *L. oregmae* populations to be sustained at higher densities when brown citrus aphids are scarce in groves. Because Singh & Hoy (unpublished) recently found that *L. oregmae* could parasitize milkweed aphids, *Aphis nerii* Boyer de Fonscolombe, on scarlet milkweed (*Asclepias curassavica* L.) in Florida, the host range of *L. oregmae* needs further analysis. Brown citrus aphid populations are maintained at very low densities in citrus groves unless abundant new growth is present. As a result, brown citrus aphid populations (and any parasitoids attacking them) in groves probably are limited to intervals when major flush cycles occur because the host range of brown citrus aphids is restricted to *Citrus* species and some Rutaceae such as lime berry (*Triphasia trifolia*), box-orange (*Severinia buxifolia* (Poir.)), and orange jasmine (*Murraya paniculata* (L.)), which are used as landscape hedges in south Florida (Tsai 1998).

Quantification of the impact of *L. oregmae* and *L. testaceipes*, in combination with the role of endemic predators and pathogens, on brown citrus aphid populations in Florida remains to be determined because *L. oregmae* was still in the process of colonizing suitable aphid species on citrus, vegetables, ornamentals, and weeds during this study. Thus, the impact of *L. oregmae* on brown citrus aphid populations, and on other aphid species on other host plants in Florida remains to be resolved.

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