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Rearing and biology of the decapitating fly *Pseudacteon bifidus* (Diptera: Phoridae): a parasitoid of tropical fire ants

Sanford D. Porter[1,], and Robert M. Plowes[2]*

Abstract

The small decapitating fly, *Pseudacteon bifidus* Brown and Morrison (Diptera: Phoridae), is a parasitoid of the tropical fire ant, *Solenopsis geminata* (F.) (Hymenoptera: Formicidae). This fly is of interest as a potential self-sustaining biocontrol agent because tropical fire ants are invasive pests throughout the world's tropics, especially on islands of the Pacific. The objective of this study was to develop methods for mass rearing *P. bifidus* and to study related aspects of its biology. The flies used in this study were collected near the Nueces River north of Catarina, Texas, USA. We found that *P. bifidus* parasitizes minor workers with an average head width of 0.71 ± 0.10 mm (0.59–1.15, range). The sex ratio of adult flies was moderately skewed to males (58:42%), with males slightly more likely to emerge from the smallest hosts and females from the largest ones. The average generation time was 30 d at 27.6 °C. Average larval development time was 14 d at 27.6 °C, but the pattern was highly skewed with a mode of 11 d and about 15% of individuals in a long tail of slow developing larvae, which extended out to at least 41 d. Male pupae emerged faster than female pupae (0.8 d, 23.5 °C). Unlike other *Pseudacteon* species, adult females were not ready to oviposit until 8 to 24 h after eclosure. We were able to rear 9,500 ± 2,800 flies per generation primarily by modifying preexisting rearing procedures (1) to provide adults access to water and sugar water so they could live longer, (2) by extending access to hosts for 1 to 2 extra d, and (3) by avoiding reuse of host colonies with poor rates of parasitism. Labor costs were decreased by rearing in discrete generations and the use of an attack box with automatic temperature, humidity, lighting, and mechanical controls that allowed flies to emerge, mate, and parasitize hosts without the need for constant management. The success of these rearing efforts provided a foundation for subsequent studies of *P. bifidus* host specificity and host suitability.

Key Words: *Solenopsis geminata*; sex ratios; development rates; host preferences; biological control

Resumen

La mosca decapitadora *Pseudacteon bifidus* Brown y Morrison (Diptera: Phoridae) es un parasitoide de la hormiga de fuego tropical, *Solenopsis geminata* (F.) (Hymenoptera: Formicidae). Esta mosca es de interés como un agente potencial de biocontrol autosostenible, ya que la hormiga de fuego tropical es una plaga invasora en zonas tropicales a nivel mundial, especialmente en las islas del Pacífico. El objetivo de este estudio fue desarrollar métodos para criar masivamente *P. bifidus* y estudiar aspectos relacionados con su biología. Las moscas utilizadas en este estudio se recolectaron cerca del Río Nueces al norte de Catarina, Texas, USA. Encontramos que *P. bifidus* parasita las trabajadoras menores con un ancho de cabeza promedio de 0,71 ± 0,10 mm (rango: 0,59–1,15). La proporción sexual de los adultos fue moderadamente sesgada hacia los machos (58:42%), con los machos emergiendo con una mayor probabilidad de los hospederos más pequeños y las hembras de los más grandes. El tiempo promedio generacional fue de 30 d a 27,6 °C. El tiempo de desarrollo larval promedio fue de 14 d a 27,6 °C, pero el patrón de desarrollo estaba muy sesgado con una moda de 11 d y alrededor del 15% de los individuos con una asimetría positiva de por lo menos 41 d. Los machos emergieron de las pupas más rápido que las hembras (0,8 d, 23,5 °C). A diferencia de otras especies de *Pseudacteon*, las hembras adultas se encuentranno estaban listas para ovipositar entre las 8 y 24 h después de emerger. Se pudieron producir 9.500 ± 2.800 mosquitas por generación modificando principalmente procedimientos de crianza preexistentes (1) proveyendo a los adultos acceso a agua y agua azucarada para que pudieran vivir más tiempo, (2) extendiendo el acceso a los hospederos por 1 para 2 días adicionales y (3) evitando la reutilización de colonias hospederas con tasas bajas de parasitismo. El costo de la mano de obra disminuyó al producir generaciones discretas y al usar una caja de ataque con la temperatura, humedad, iluminación y controles mecánicos automatizados que permitieron a las mosquitas emerger, aparearse y parasitar sus hospederos sin la necesidad de una manipulación constante. El éxito de estos esfuerzos para criar *P. bifidus* proporcionaron una base para estudios posteriores de especificidad y aceptabilidad del hospedero.

Palabras Claves: *Solenopsis geminata*; proporción sexual; tasa de desarrollo; preferencia de hospederos; control biológico

The fire ant decapitating fly, *Pseudacteon bifidus* Brown and Morrison (1999; Fig. 1) (Diptera: Phoridae), is a small native parasitoid of tropical fire ants (*Solenopsis geminata* [F.]) (Hymenoptera: Formicidae) in Texas and neighboring regions of Mexico (Plowes et al. 2009). Data reported as *P. crawfordi* in Morrison et al. (1997), "Sp. A" in Morrison & Gilbert (1998), and Morrison et al. (1999b) are actually for *P. bifidus* (Brown & Morrison 1999). *Pseudacteon bifidus* is one of about 20 species of *Pseudacteon* decapitating flies known to parasitize tropical fire ants, and

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Fig. 1. Lateral view of male (left) and female (right) *Pseudacteon bifidus* decapitating flies. The inset shows the tripartite lobes of a fresh female oviscape or external ovipositor prior to the middle lobe being obscured by an opaque white membrane after preservation in alcohol, which gives preserved flies the appearance of a bifid or bipartite external oviscape. Also, note that male antennae are elongated and lack the aristae found on the tips of female antennae. Adult flies range from 0.72 to 0.88 mm in length (Plowes et al. 2009).

is grouped with 4 small species having short forked oviscapes (Plowes et al. 2009). *Pseudacteon bifidus* is about the same size as *Pseudacteon curvatus* Borgmeier (Morrison et al. 1997; Brown & Morrison 1999), an abundant introduced parasitoid of red imported fire ants (*Solenopsis invicta* Buren) in the United States (Callcott et al. 2011).

Pseudacteon bifidus females parasitize minor *S. geminata* workers (Morrison et al. 1997; Morrison & Gilbert 1998). Oviposition attempts generally only last a fraction of a s and a single egg is injected into the thorax of host workers. The resulting larvae and puparia are typical of other *Pseudacteon* species (Porter 1998). About 26% of oviposition attempts are successful and development from egg to adult requires about 29 d at 30 °C (Morrison et al. 1997). Morrison et al. (1999b) reported that *P. bifidus* flies in central Texas are not active at temperatures below 20 °C or from Dec until Apr. They also reported that, unlike some species, *P. bifidus* males are not attracted to fire ant hosts. Females apparently are capable of dispersing at least 0.5 km (Morrison et al. 1999b).

Parasites of tropical fire ants, like *P. bifidus,* are of interest as potential biological control agents because tropical fire ants are invasive pests throughout most of the world's tropics, especially in the Pacific (Wetterer 2011; Gotzek et al. 2015). The primary objectives of this study were to develop methods for mass rearing *P. bifidus* and to investigate related aspects of this fly's life history including host requirements, sex ratios, and development times of immature stages. Rearing methods described in this paper were based on techniques developed to rear a series of *Pseudacteon* parasitoid species of the red imported fire ant (Porter et al. 1995a, 2013; Vogt et al. 2003).

Materials and Methods

INITIAL COLLECTION

The *P. bifidus* flies used in this paper were collected along a ranch road about 100 m south of the Nueces River in Dimmit County about 16 km north of Catarina, Texas (28.5005°N, 99.6276°W). Collections were made on 1 May 2014 by setting out 10 trays (28 × 38 × 10 cm) with *S. geminata* workers and brood from Gainesville, Florida, USA, for 4 to 5 h in areas where *P. bifidus* was common. An opaque trap nest (15 × 6 × 2 cm) with plugs of moistened plaster in the top was placed in each tray and moved from one end of the tray to the other every 5 to 10 min so that workers continuously transported their brood back and forth across the tray while flies attacked (see Supplement for photo: http://purl.fcla.edu/fcla/entomologist/browse or http://www.ars.usda.gov/saa/cmave/ifahi/bifidus).

LABORATORY MASS-REARING

The rearing procedures for *P. bifidus*, which are detailed below, were initially similar to those used for *Pseudacteon cultellatus* Borgmeier (Porter et al. 2013 and its Supplement), except the protocols used to minimize *P. curvatus* contamination were not needed because *P. curvatus* does not normally parasitize *S. geminata* workers. Emergence, mating, and host parasitization occurred in 2 large attack boxes (2 × 1 × 0.6 m) with clear plastic tops similar to the box described by Vogt et al. (2003) except our boxes had 4 sets of reach-in sleeves and gloves along the sides rather than access doors (see Supplement). Our boxes were illuminated by 4 cool-white 48 inch fluorescent tubes (34

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W). Ants in each box continuously traveled back and forth in 8 trays (40 × 28 × 8 cm) by automatically lifting and lowering refuge cups every 12 min for the ants to hide under (Supplement for Porter et al. 2013).

Between 9:00 AM and 7:00 PM, temperature in the attack boxes was regulated at 27.0 ± 0.5 °C by heat from two 300 W halogen lamps controlled by a thermostat. Temperature in attack boxes gradually fell to room temperature (about 23.5 °C) each d when lights were turned off by timers. Relative humidity in the attack box was maintained at 87 ± 4% RH by continuously circulating moist air into the attack box from a lower bay containing a vaporizer controlled by a humidistat (see Supplement for Porter et al. 2013).

Newly emerged adult decapitating flies entered the attack box from a side chamber that contained trays of puparia. These trays were switched back and forth between attack boxes every 3 to 4 d. Ant workers were retained in the attack boxes for an additional 1 to 2 d (4–6 total) to provide hosts for the remaining female flies until most of the females had died. The primary benefit of swapping between boxes was that it allowed the ant workers to be extracted without the risk of large numbers of female flies chasing after the workers when they were removed.

To save effort, fly attacks were conducted only for 16 to 20 d for each generation, thus giving 12 to 15 d without fly attacks. To facilitate fitting in this window, the last several d of pupal production were sometimes accelerated by placing them at 27.5 °C rather than room temperature (about 23.5 °C).

Each attack box contained 4 sugar wicks (10% sugar by weight) and 2 water wicks affixed to the top by Velcro where mating flies would be likely to land on them (Supplement). Wicks consisted of rolling 2 sheets of lab tissue together and inserting them into the cut off end of a 10 ml plastic centrifuge tube. Sugar and water wicks were changed out every 3 to 4 d.

We collected *S. geminata* workers from the area around Gainesville, Florida (Supplement), as hosts for the flies. Workers were used without sieving out larger workers because *P. bifidus* prefers small workers and, unlike *S. invicta*, larger workers are a relatively small proportion of *S. geminata* colonies. Each of the trays in attack boxes described above usually contained 1.0 g of ant workers and 1.0 to 1.5 g of ant brood. Before being put into the attack box, workers were allowed to bond with brood (from other colonies) for 30 min (Vogt et al. 2003). Brood was needed to ensure that workers did not freeze up when exposed to fly attacks. We used *S. geminata* brood for the first several fly generations, but switched to *S. invicta* brood after trials showed that it was readily accepted by *S. geminata* workers and did not interfere with rearing efforts. Most of this brood was removed after workers were removed from attack boxes.

After exposure to fly attacks, workers were placed in holding boxes (25 × 23 × 8 cm) with vented lids and moisture blocks that maintained relative humidity around 95% (Supplement for Porter et al. 2013). Dead workers with pupariating larvae were collected 6 d a wk from 8 to 15 d, after attacks, and placed on moist plaster trays stored in high humidity holding boxes at room temperature (about 23.5°C). Several d later, we made one-half or one-fourth count estimates of pupal production on each tray. For parts of fly generations 8 to 10, we segregated dead workers from different colonies on plaster trays so that variability in parasitism rates among host colonies could be determined.

Rearing and managing the *P. bifidus* fly colony required the full-time efforts of about 2 technicians plus backup and scientific oversight (SDP) to troubleshoot problems. Included in the Supplement for this paper are task lists for weekdays and weekends, a weekly quality control inspection sheet, and several check sheets for critical tasks along with photos of equipment and procedures not included in the Supplement for Porter et al. (2013).

HOST SIZE PREFERENCES

Different species of decapitating flies attack different sizes of fire ant workers (Porter 1998) and sometimes the size of its host determines the sex of the fly (Morrison et al. 1999a). In order to determine what sizes of tropical fire ants *P. bifidus* females select as hosts, and whether host size affects the sex of progeny, we selected 61 puparia from a 5 d period in Jun 2014 and 100 puparia from a 10 d period in Jul 2014. Puparia were selected by a predetermined algorithm so that they were representative of other puparia at those dates. Subsequently, a supplemental sample also was taken of the largest and smallest ant heads containing fly puparia. All puparia were placed on yellow sticky cards (Alpha Scents, West Linn, Oregon, USA; www.alphascents.com) and held in high humidity holding boxes until they all emerged. We recorded the date and sex of newly emerged flies trapped on the sticky cards daily. Host head widths were determined with an ocular micrometer. Head widths of a representative sample of 161 unparasitized workers were determined with a wedge micrometer (Porter 1983).

DEVELOPMENT TIMES OF IMMATURE STAGES

Knowing the timing and duration of immature and adult stages helps to organize laboratory rearing activities. In order to determine the time from oviposition to pupariation, we allowed flies to emerge into a large attack box for 2 d. Oviposition attacks began on the afternoon of the first d then allowed to continue through midday on the fourth d. This was repeated 3 times using workers from 2 colonies. Potentially parasitized workers were held at 27.6 ± 0.7 °C in humidified boxes. Dead or dying workers were collected daily for the first 2 wk, then every second d thereafter. Dead workers were placed on moist plaster trays and inspected for fly puparia on the second d. In a test to determine if temperature affected successful larval development, half of parasitized workers with 3rd generation flies were held at 23.5 °C and the other half were held at 27.6 °C.

In order to determine the duration of the puparial stage, we allowed flies to attack 9 groups of ants from different colonies for 3 to 4 d each. Dying ant workers were collected daily as noted earlier and held at 23.5 ± 1.0 °C. We then collected a subsample of 15 to 30 puparia from each worker group, distributed proportionately according to the number of fly larvae pupariating on each date. This was conducted to assure a representative sample of individuals across dates of pupariation. As noted above, these puparia were placed on yellow sticky cards so we could determine the sex of newly emerged flies and record the date of emergence. Puparia also were collected from 5 dates and reared at 27.6 °C to determine to what extent warmer temperatures would accelerate their development.

ADULT SEX RATIOS

The sex ratio of adult flies was determined from the pupal development rate test and host size test discussed earlier. We also collected puparia from slow developing larvae in the long skewed tail (Table 1; see Fig. 3) to determine sex and emergence rates of these individuals. This was accomplished by daily collecting 2 to 3 puparia from each of 3 worker groups for 11 to 14 d after the primary pupariation peak. These puparia were handled as above.

DATA ANALYSIS

We used the *t*-test option in StatPlus:mac Pro software (AnalystSoft Inc, Walnut, California, USA; v6, http://www.analystsoft.com/en/) to determine significant differences between sample means. The option for unequal variance was used when necessary. The ANOVA option

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Table 1. The sex ratio of *Pseudacteon bifidus* adults is moderately skewed toward males in representative samples. The values below show the proportion and percentages of males in test samples and the probabilities of observed deviations from a 1:1 sex ratio.

	Males		
Pupal Source	Proportion	Percent	Probability[a]
Representative Samples			
Host Size Test	85/142	59.9	0.023
Development Test	86/153	56.2	0.145
Above Combined	171/295	58.0	0.007
Selected Samples			
from slow-growing larvae	32/58	55.2	0.512
large hosts[b] (≥0.88 mm)	4/20	20.0	0.012
small hosts[b] (≤0.66 mm)	29/39	74.4	0.0034

[a]2-tailed exact binomial probability.
[b]Values show host head widths.

was used to compare fly development times in hosts from different ant colonies. We used 2-tailed exact binomial tests to determine whether sex ratios differed significantly from 1:1 (VassarStats: Richard Lowry; http://vassarstats.net/binomialX.html).

Results

INITIAL COLLECTION

A total of 2,900 *Pseudacteon* puparia were collected from the 10 trays of *S. geminata* that had been exposed to fly attacks (Fig. 2). Flies emerged from 78% of puparia. Approximately 95% of these flies were *P. bifidus*, 4% were *P. catarinae* Plowes et al., and 1% were *P. hippeus* Plowes et al. The *P. bifidus* males and females (Fig. 1) that emerged from these puparia were transferred into the large attack boxes to establish the laboratory colony used in the studies below. Efforts also were made to rear the *P. catarinae* flies in the lab using smaller attack boxes (see Supplement), but they were not successful.

LABORATORY MASS-REARING

The second generation of *P. bifidus* produced 3,280 puparia (Fig. 2) with an emergence rate of 77%. Subsequent generations (3–17) averaged 9,500 ± 2,800 (± SD) puparia with an average emergence rate of 89 ± 3%.

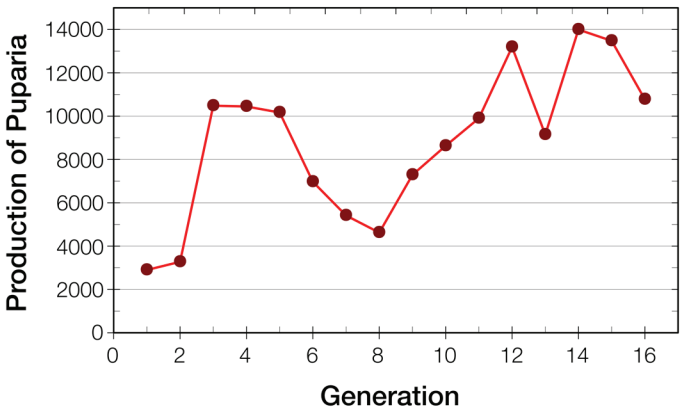

Fig. 2. Production of *Pseudacteon bifidus* puparia in our laboratory colony plotted by generation. The average generation time in our lab was about 32 days.

From the 6th to 8th generations (Oct–Dec), production gradually declined to 4,600 puparia (Fig. 2). The cause of this decline is unknown. Seasonal variability in the suitability of field workers is one possible explanation. However, we also found that workers from different *S. geminata* colonies varied considerably in their suitability as hosts for the flies. Colonies from the lowest producing quartile produced only about 10% of the flies as those in the highest producing quartile (8.2 versus 78.2 fly puparia per tray per d, n = 28). Preliminary observations indicated that inter-colony differences likely resulted because workers from some colonies were more attractive to ovipositing females while workers from other colonies appeared to be poor hosts for developing larvae. Workers reared in the laboratory performed more poorly as hosts than workers from field colonies (20.9 versus 45.8 puparia per tray per d, n = 9 and 19, 2-tailed *t*-test (unequal variance), $t = 3.11$; df = 25; $P = 0.0046$); however, the 2 worst producing colonies were from the field. We found that workers from some colonies were more sensitive to fly attacks than others and were considerably more difficult to keep moving between refuge cups during fly attacks. Colony differences in successful parasitism were not correlated with mean worker weight, collection locations, or emergence rates of puparia.

As with other *Pseudacteon* flies, *P. bifidus* adults emerged from their puparia only in the early morning. We found that *P. bifidus* females did not begin reliably ovipositing until about 4:00 PM or 8 to 10 h after eclosure from the puparium. Peak oviposition activity generally occurred on the second d with most female flies dying by the end of that d when they had access to hosts (SDP, unpublished data). Males appeared to live longer than females, probably because they were not attracted to fire ant workers. Mating appeared to occur in the top of the large attack box, often around black strips of velvet (about 10 cm, see Supplement) where the males seemed to congregate.

Access to sugar water and water in wicks seemed to improve survival of flies, especially males, into the second and third d (Chen & Fadamiro 2006); however, several pilot trials with only access to water wicks still produced good numbers of puparia. Honey water initially was used but was discontinued because it developed mold faster than sugar water and did not show a clear benefit. A variety of potted flowering plants also were tried in attack boxes in early generations to provide food and potential refuge sites for flies, but this practice also was discontinued because plants did not improve fly production.

HOST SIZE PREFFERENCES

The mean head width of host workers with fly puparia was 0.714 ± 0.097 mm (SD, n = 161). The mean head width of hosts, with successful puparia (0.707 ± 0.091, n =142), was smaller than the mean for hosts with puparia that failed to produce flies (0.765 ± 0.126, n = 19; 2-tailed *t*-test, $t = 2.48$; df = 159; $P = 0.014$). Head width of hosts with puparia that emerged successfully as adults ranged from 0.59 to 1.15 mm with a median of 0.69 mm. By way of comparison, median head width of non-parasitized workers was 0.67 mm with a range of 0.60 to 2.35 and a mean of 0.799 ± 0.372 mm. Females emerged from slightly larger hosts than males (0.726 ± 0.109 versus 0.695 ± 0.075, n = 57 and 85; 2-tailed *t*-test, $t = 2.02$; df = 140; $P = 0.045$).

DEVELOPMENT TIMES OF IMMATURE STAGES

Larvae in parasitized workers held at 27.6 °C began pupariating as soon as 9 d after the first attacks and continued up to at least 41 d (Fig. 3), although some individuals likely require even longer time periods. The mean combined time for egg and larval stages was 14 d but the peak, or mode, was about 11 d after oviposition, assuming most of the attacks occurred on the second d of the tests. Slow developing individ-

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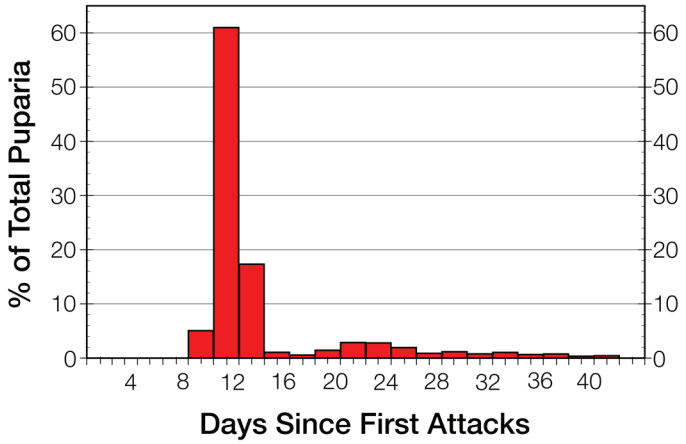

Fig. 3. Histogram showing the distribution of development periods from oviposition to pupariation of the decapitating fly *Pseudacteon bifidus* at 27.6 ± 0.7 °C. Percentages were calculated from 1,766 fly puparia resulting from 3 trials with ants from 2 colonies.

uals formed a long tail on the frequency distribution plot (Fig. 3). This tail contained about 15% of total puparia with a hint of a second peak around 21 to 22 d (Fig. 3). This pattern was very similar for all 3 trials. We found that puparia selected from the long tail of this distribution had an emergence rate of 80.6% (58 of 72) and a slightly male-biased sex ratio (55.2%, Table 1). Parasitized workers from the 3rd generation raised at 23.5 °C and 27.6 °C produced similar numbers of puparia (5,369 and 5,113, respectively).

At 23.5 °C, female puparia (n = 53) in our lab colony required an average of 22.4 ± 1.3 d (SD) for the flies to emerge, while male puparia (n = 74) required 0.8 d less (21.6 ± 0.8 d; $t = 4.32$; df = 125; $P < 0.0001$; 2-tailed *t*-test; Fig. 4). The range was 20 to 27 d for female puparia and 19 to 24 d for male puparia (33 of 186 puparia failed to emerge and were not sexed). Development rates did not differ significantly among the 9 colony host groups (ANOVA, $P > 0.3$). Total development time from egg to adult at the temperatures used in our rearing setup was about 33 d (calculated on modal data) and 29 to 70 d after summing the extremes. By way of comparison, mean developmental time for puparia at 27.6 ± 0.7 °C was 16.0 ± 0.5 d (± SD, n = 31, range 15–17) or about 6 d faster than at 23.5 °C. Total development time at 27.6 °C would be about 30 d (adding mean times for egg to puparia and puparia to adult).

ADULT SEX RATIOS

The sex ratio of adult *P. bifidus* appears to be moderately male biased (Table 1). In the host size test, flies emerged from 88.2% (142 of 161) of the representative sample where 59.9% were males. In the developmental time test, flies emerged from 82.3% (153 of 186) of the puparia where 56.2% were males (Table 1). The combined percentage of males for both tests was 58.0%. A selection of the largest parasitized ant heads (≥ 0.88 mm) produced 20.0% males while a selection of the smallest heads (≤ 0.66 mm) produced 74.4% males (Table 1).

Discussion

REARING A NEW SPECIES OF DECAPITATING FLY

Pseudacteon bifidus is the first decapitating fly to be successfully cultured with the tropical fire ant, *S. geminata*. Rearing decapitating flies is difficult and successful rearing (Pesquero et al. 1995; Porter et

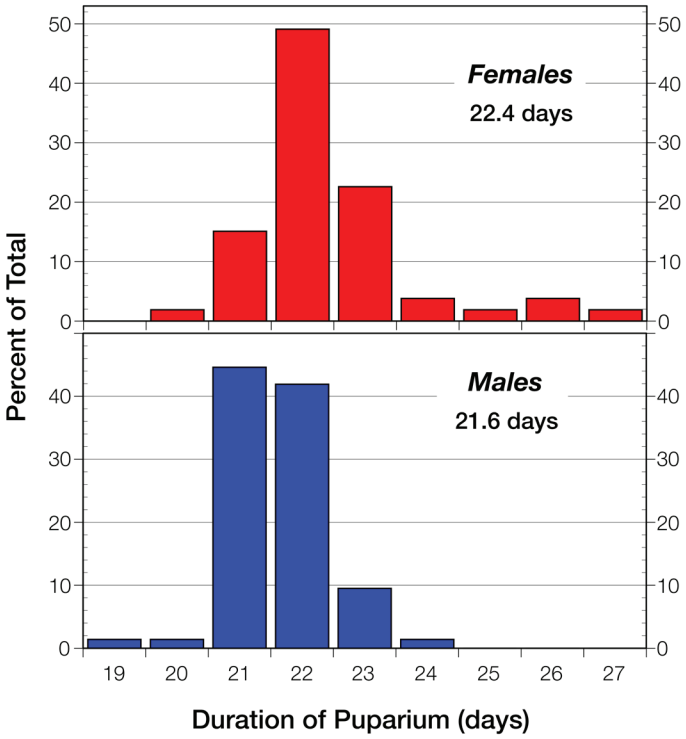

Fig. 4. Histograms showing the duration of the puparial stage of male and female *Pseudacteon bifidus* decapitating flies at 23.5 °C, as determined by the time from pupariation until emergence of the adult fly. Percent distributions were calculated from 53 female and 74 male flies which emerged from puparia sampled from 9 host colonies

al. 1995b, 1997, 2013; Gilbert & Patrock 2002; Vogt et al. 2003) is usually part art and part science. Trying to culture a new species of *Pseudacteon* fly is difficult because the initial number of founding individuals is usually small. Consequently, any rearing problems encountered may result in rapid decline of flies in subsequent generations which, in turn, can swiftly jeopardize establishment of a colony. The problem with losing a new colony is that it likely means trying to arrange another field trip, waiting for another season, getting more funds, new visas, and perhaps new import/export permits. Practitioners of many classical biological control programs will relate to this stress (Cameron et al. 1993; De Clerck-Floate et al. 2008; Paynter et al. 2016). If rearing problems can be remedied fast enough, the colony can begin to grow, at least until new problems develop. Sadly, our efforts to culture a number of *Pseudacteon* species including *P. nocens*, *P. nudicornis*, *P. obtusitus*, *P. catarinae*, and biotypes of *P. litoralis* from Formosa, Argentina, and Rio Claro, Brazil, so far have failed (Plowes et al. 2012; SDP and RMP, unpublished data).

SOLVING REARING PROBLEMS

We found that knowing the basics of phorid fly biology (Disney 1994; Porter 1998; Consoli et al. 2001; Wuellner et al. 2002) and outlining possible sources of problems followed by strategic testing for improvements were vital to rearing success. Nevertheless, rearing difficulties usually come with too many possible causes for carefully controlled testing and often require proceeding with sets of educated guesses. This was certainly the situation for rearing *P. bifidus*.

Our first crisis was that we had hundreds of flies emerging in the attack box, but no attacks, and only 5 to 6 d to solve the problem before all of the first generation flies had emerged and died. Every other species, which we had successfully reared, both mated and attacked host

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ants within a few h of emerging (Porter 1998). However, this current study revealed that *P. bifidus* usually required 8 to 24 h before females were ready to attack hosts. Additionally, male pupae emerged about a d before female pupae (Fig. 4). The net result was a considerable number of newly emerged flies during the first couple of d of the first generation that were not doing much. At first, we feared that some Florida *S. geminata* workers might not be attractive or that conditions were inappropriate for mating. Fortunately, the 2-fold solution to this first crisis was to leave the flies in the attack box an extra 1 to 2 d so that more females could emerge and mature, and secondly, to make a greater effort to provide flies with both sugar water and water soaked wicks so they would live long enough to mate and oviposit.

Our second major crisis occurred during generations 6 to 8 when puparial production fell from over 10,000 per generation to under 5,000 (Fig. 2). The initial decline did not concern us until it became a trend. At that point, we carefully reevaluated our rearing protocols and examined our environmental loggers for signs of problems (see rearing checklists and quality control inspection sheet in Supplement). For example, puparial emergence rates < 70% would have triggered fears that relative humidity was too high (condensing conditions around 100%) or too low (< 85%). Improper humidity is a likely cause for poor emergence rates reported in a number of earlier studies (Morrison et al. 1997; Porter et al. 1997; Folgarait et al. 2002a, b) because proper conditions consistently produce emergence rates of 75 to 95% (Vogt et al. 2003; also see results section above). Fortunately, the emergence rates in generations 6 to 8 remained high (86–91%), but unfortunately, we were unable to pinpoint other problems with known solutions. Consequently, we began monitoring the performance of individual host colonies and found that their suitability could vary by as much as an order of magnitude. Morrison et al. (1999b) had reported that some *Solenopsis* colonies are better hosts than others. The causes of these differences were not established, although seasonality or intrinsic colony differences associated with attractiveness, defensive behavior, or physiological suitability for developing larvae are all likely possibilities. Also, workers from laboratory colonies were not as good as workers from field colonies. However, field colonies also varied widely in their suitability as hosts. To mitigate the possibility of using only 1 to 2 bad colonies in an attack box, we used *S. geminata* workers from 3 to 4 different field colonies after the 8th generation. Also, extra unused workers from poorly performing colonies were discarded and not used as hosts in subsequent attack runs. Fortunately, production improved in subsequent generations after these changes were implemented (Fig. 2).

HOSTS, SEX RATIOS, AND IMMATURE STAGES

We found that female *P. bifidus* oviposited in minor workers of the tropical fire ant (mean head width: 0.71 ± 0.10 mm, range: 0.59–1.15 mm). Morrison et al. (1997) and Morrison and Gilbert (1998) previously reported mean host head widths from 0.74 to 0.80 mm, depending on the mean size of workers in host colonies. In other words, all minor workers are potential hosts, while some medium-sized workers and all major workers are apparently too large. We found a weak but significant tendency for females to emerge from larger hosts than males (4.5% larger head width), principally because the smallest and largest hosts tended to be male and female biased, respectively. This is the smallest significant sex-linked difference reported between hosts of male and female *Pseudacteon* flies. Two small *Pseudacteon* species that parasitize *S. invicta* (*P. curvatus* and *P. cultellatus*) do not show sex-linked host-size differences (Folgarait et al. 2002a; Chirino et al. 2009); however, most of the larger species do show differences (Morrison et al. 1999a; Folgarait et al. 2006). It is unknown whether the apparent host size-sex linkage in *P. bifidus* is related to differential mortality or is a case of environmental sex determination as appears to be the case with the larger *Pseudacteon* species (Morrison et al. 1999a).

We found that sex ratios in *P. bifidus* were modestly male biased (58%, Table 1). By way of comparison, the sex ratios for *P. curvatus* and *P. cultellatus* are about even (1:1) (Folgarait et al. 2002a; Chirino et al. 2009) while *P. obtusus* is even (Calcaterra et al. 2005) or a bit female biased 52 to 63% (Porter & Calcaterra 2013; Porter et al. 2013). In contrast, *P. nocens* is strongly male biased in laboratory tests (75%, Folgarait et al. 2006). *Pseudacteon tricuspis* (Diptera: Phoridae) is moderately to strongly male biased with field collected flies: 78% (Calcaterra et al. 2005), 56 to 71% (Henne & Johnson 2009), 73% (Morrison & Porter 2005), and 57 to 64% (Porter & Calcaterra 2013; Porter et al. 2013). Interestingly, Chirino et al. (2012) reported that male-biased sex ratios occurred when multiple female *P. tricuspis* competed for hosts during laboratory tests, but the sex ratio was about even when females were not in competition. Perhaps some of the variability in *P. tricuspis* sex ratios noted above is related to variability in abundances and competition in the previous generation.

Developmental rates of *P. bifidus* immature stages (Figs. 3, 4, and results; Morrison et al. 1997) were more rapid than other *Pseudacteon* species at comparable temperatures. For example, total development time for *P. bifidus* at 27.6 °C averaged about 30 d compared with 32 to 33 d for *P. curvatus* (Chirino et al. 2009), and 34 to 37 d for *P. obtusus* (Folgarait et al. 2005). Other *Pseudacteon* parasitoids of fire ants required 38 to 53 d to complete development at 27 to 28 °C (Folgarait et al. 2002b, 2006; SDP, unpublished data). Several papers have reported that development times varied significantly depending on the species or population of the host in which they developed (Porter & Briano 2000; Folgarait et al. 2002a, 2005, 2006). We found that male puparia completed development about a d faster than female puparia (Fig. 4). However, studies with *P. curvatus*, *P. tricuspis*, *P. cultellatus*, *P. obtusus*, and *P. nocens* did not find significant sexual differences in puparial development times (Folgarait et al. 2002a, 2005, 2006; Chirino et al. 2009, 2012). We did not determine if larval development times differed between *P. bifidus* sexes, but the studies noted above did not report that larval development rates were affected by sex, except perhaps for *P. nocens* which showed a significantly longer time for females at 28 °C but not at 22 °C (Folgarait et al. 2006).

The long tail of slow developing *P. bifidus* larvae (Fig. 3) appears to be a character of all *Pseudacteon* species that parasitize fire ants (SDP and RMP, unpublished data). For *P. bifidus*, the proportion in the tail (15%; Fig. 3) is similar to *P. curvatus* (19%; SDP, unpublished data), but much less than the proportion for *P. tricuspis* (39%; Morrison & Porter 2005). The proximal cause of this highly skewed distribution is not known, but it clearly increases the probability that a portion of the larvae might pupariate during moister or warmer weather conditions if drought or cold weather had limited success for the primary peak. The emergence pattern for *P. bifidus* pupae was much shorter and more normal (Fig. 4) than the pattern for pupating larvae (Fig. 3), indicating that there is either a penalty or little benefit for delayed pupal development.

Morrison et al. (1999b) reported that *P. bifidus* and other *Pseudacteon* parasitoids of *S. geminata* in Texas appeared to have a winter diapause. So far, we have found no sign of a temperature induced diapause in *P. bifidus* or in other *Pseudacteon* species reared in the laboratory at a variety of temperatures. Consequently, it is uncertain whether the dearth of flies Morrison et al. (1999b) observed in the spring was due primarily to slowed developmental rates and winter mortality, or to environmental triggers of actual diapause not found in our laboratory colonies.

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Understanding the effects of temperature on development rates of *P. bifidus* and controlling rearing temperatures was beneficial because it allowed us to: (1) predict the timing of upcoming generations and rearing procedures, (2) expand the duration of the puparial stage to help segregate generations, (3) compress the duration of the larval stage so that less of the tail was lost, and (4) accelerate development of puparia at the end of a generation so that adults would emerge earlier in a narrower window. Degree-day calculations for puparial development of other *Pseudacteon* species also has allowed accurate modification of adult emergence to match needed field release dates (SDP, unpublished data) or to concentrate the number of individuals available for field releases (Thead & Streett 2006).

NOTES ON REARING EFFICIENCY AND LABOR COSTS

Culturing *P. bifidus* parasitoids in the laboratory was important because it provided sufficient individuals for host testing (Porter et al. 2018) and potential inoculative field releases. Nevertheless, culturing these flies was an expensive and time consuming effort. We employed a number of strategies to reduce these costs. First we used highly automated attack boxes that used timers to regulate temperature, humidity, lighting, and pneumatic motors which raised and lowered refuge cups so that the ants were exposed to fly attacks. These automatic systems and the ability of the flies to emerge into the attack boxes and mate allowed rearing to continue over the weekends with little or no oversight. Well-trained technicians were a second key to efficiency. Standard rearing protocols and quality control checks limited rearing mistakes, and technicians were encouraged to report problems and suggest improvements to protocols. We used subsampling procedures (usually quarter counts) to speed daily estimates of pupal production. Limiting *P. bifidus* rearing to discrete generations was important because it meant that we had about 12 to 15 d for each generation when attack boxes were not running and about the same amount of time when pupariating larvae did not need to be collected. Preliminary tests that demonstrated *P. bifidus* host specificity in the field (Porter et al. 2018) were important because they justified further efforts to establish and maintain *P. bifidus* in the laboratory so that more comprehensive tests could be completed.

We were able to complete laboratory host specificity testing after 5 mo because of good rearing success. The major delay we ran into was obtaining tropical fire ants from various Pacific islands for host suitability testing (about 8 additional mo). This delay was partly due to the time required to obtain import/export permits for importing live fire ants, and partly due to the time needed to arrange for collection and successful shipment of test ants. Sadly, these delays were the most expensive part of our rearing efforts.

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