Phenology of *Pseudacteon* spp. (Diptera: Phoridae) flies in Alabama

by

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A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
December 13, 2014

Keywords: *Pseudacteon curvatus*, *Pseudacteon litoralis*, *Pseudacteon tricuspis*, phorid
fly, decapitating fly, *Solenopsis invicta*, fire ant, biological control

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Abstract

Phorid flies, *Pseudacteon* spp. (Diptera: Phoridae), are one of many biological control agents of *Solenopsis* spp. (Hymenoptera: Formicidae) fire ants in their native South America. More than twenty species of phorid flies are known to parasitize fire ants, of which, five species (*Pseudacteon cultellatus*, *Pseudacteon curvatus*, *Pseudacteon litoralis*, *Pseudacteon obtusus*, and *Pseudacteon tricuspis*) have been released into Alabama. Three species, *P. curvatus*, *P. litoralis*, and *P. tricuspis* are currently established in Alabama and were the focus of this dissertation.

Diurnal flight patterns of *P. curvatus, P. litoralis* and *P. tricuspis* were monitored in Alabama in 2010, and again from 2012-2013. During the study periods, the *Pseudacteon* species were co-established. These data were compared to data collected in 2002, when both *P. curvatus* and *P. tricuspis* were established but were not yet inhabiting the same areas. Both *P. curvatus* and *P. tricuspis* were active mid-day. The data collected in this study provide evidence that *P. curvatus* may be more successful than *P. tricuspis* when the species are co-established.

Seasonal abundance of *P. curvatus* and *P. tricuspis* was monitored between 2012 and 2013. This study was important in order to determine any differences from the patterns observed in South America. By finding the highest periods of abundance, researchers will be able to target that time of year for future studies on *Pseudacteon* phorid flies. A total of 7,284 *P. curvatus* individuals were collected during this study.

Pseudacteon curvatus was most abundant between June and August of 2012. *Pseudacteon tricuspis* was found only in June and September of 2012, and a total 23 *P. tricuspis* individuals were collected over these dates.

Alabama is the only state where *P. litoralis* is established, but it is only found in high numbers in a remote location of Wilcox County. The location of this species makes it difficult to study without significant funding. In order to monitor the outward spread of this species, two new trapping methods were tested against the currently used method. If successful, these new methods would allow for data to be collected in a more efficient manner. The tray trapping method, which is the method currently used to collect for *P. litoralis* in the field, was more successful than both new trap types tested.

Percent parasitism data were collected between July and October 2013 in Alabama where *P. curvatus* and *P. tricuspis* were co-established. During this study, only *P. curvatus* were collected. A mean parasitism rate of 0.059% was observed for *P. curvatus*, which was similar to the observed rates in Florida when only *P. tricuspis* was established.

These studies provide important and unique observations of the interactions among *Pseudacteon* spp. once co-established in the United States. The data suggest that current protocols may need to be altered in order to increase the likelihood of success of the biological control program.

Acknowledgments

This work would not have been possible without the help of many people. As the African proverb states, "It takes a village to raise a child." These words seem to sum up how I feel as I finish this degree. None of this would be possible if not for my boss, who I also call my friend, Fudd Graham. He always encouraged me to pursue an advanced degree, even when I was adamant that I would never get a degree in Entomology, because "bugs are yucky." I would never have imagined that after working with him for eight years that I would now find insects fascinating, and not at all disgusting.

I want to thank my major professor, Kathy Flanders, who is more like family than just a member of my committee. She put a lot of faith in me to get my work done independently and always had helpful tips and advice along the way. Fudd and Kathy are my "fire ant family," they took me under their wings and helped me achieve more than I ever thought possible.

My family has always supported me in anything that I wanted to do, and this held true when I told them that I was going to get my degree in Entomology. Although, I'm now introduced as the "weird child that likes bugs," I will always be thankful for their unwavering support. My husband, Joseph, has been with me through everything. He has continued to support and encourage me to get this work done. He was often met with continual complaints, a messy house and no thoughts on what we were going to have for

dinner. He never complained, and for that, I am extremely grateful. We worked together to get through vet school and a Ph.D. program, and it feels great to be done!

I want to thank my committee: David Held, Henry Fadamiro and Nannan Liu. I was lucky to have you all as professors, and get to work with you through my job. I feel blessed to have had the opportunity to get to work with you and I hope to continue to work with you in the future. Thanks are also due to Julie Howe, my university reader. Thanks for taking the time to read my dissertation and give your insights.

I also want to thank Edzard Van Santen and Kira Bowen. They were a tremendous help with my statistics for my *P. litoralis* trap study and my parasitism study, and for that I am extremely grateful.

Finally, I wish to thank Phil and Daria Story and Walt and Peggy Prevatt, the landowners, for allowing me to use your facilities for this research. I could not have asked for better people to work with, thanks!

I would like to finish with the best words of advice I have received throughout this process. Fudd once told me, "The day that you stop learning, is the day you need to retire." I hope that I will continue to learn for many years to come.

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I. Introduction

FIRE ANTS

Imported fire ants, *Solenopsis* spp. (Hymenoptera: Formicidae), are highly aggressive, invasive pests that are native to the flood plains of Argentina and Brazil in South America (Vander Meer & Lofgren 1990). Both the black imported fire ant, *Solenopsis richteri* Forel, and the red imported fire ant, *Solenopsis invicta* Buren, were accidentally introduced into the United States prior to 1950. Since their introductions, the two species spread rapidly (Imported Fire Ant eXtension Community of Practice 2014) and today pose a serious threat to the health of the public, the economy and the environment.

Both the black and the red imported fire ants were introduced either through the ballasts of ships (Vinson 1997) or through infested fruit cargo (George 1958) from ships travelling from South America. The black imported fire ant was first identified in the United States in 1929 (Löding 1929) and is thought to have been introduced through the port of Mobile, Alabama around 1918 (Creighton 1930). The black imported fire ant that infests the United States originated just north of Buenos Aires, Argentina (Vander Meer & Lofgren 1990). The red imported fire ant is believed to have entered the United States via the port of Mobile, Alabama between 1933 and 1945 (Callcott & Collins 1996). The red imported fire ant that infests the United States originated in southwestern Brazil (Vander Meer & Lofgren 1990). The two fire ant species do not readily hybridize in their native South America, likely due to limited geographical contact between the species (Tshinkel 2006). However, the species are in continual contact with one another in the U.S., which allows for frequent hybridization.

In 1958, the federal imported fire ant quarantine was established to help limit the artificial spread of fire ants and to develop control strategies for agricultural producers. Since the establishment of the quarantine, imported fire ants continue to spread approximately 2.4 million ha per year (Callcott 2002). Today, the species infest approximately 172 million ha of the Southeastern U.S., much of Texas, and parts of Oklahoma, New Mexico and California (Imported Fire Ant eXtension Community of Practice 2014). Imported fire ant spread is limited to the north due to climatic variables associated with freezing temperatures during the winter months (Porter 1998; Callcott & Collins 1996). Most of the imported fire ant infested areas contain the red imported fire ant or the hybrid. The black imported fire ant has been pushed to the far northern areas of the quarantine zone, thus only being found in northern Mississippi, northwestern Alabama, and southern Tennessee (Imported Fire Ant eXtension Community of Practice 2014). This is attributed to their less aggressive nature and their ability to tolerate slightly colder temperatures (Lofgren et al. 1975).

Populations of imported fire ants pose serious health and economic risks in areas where they are established. Fire ants cause approximately $7 billion in damage in the United States each year (Lard et al. 2001). These costs are associated with damage, repair and control of the invasive pests. Damage is known to occur to electrical equipment (Drees 1998), agricultural equipment such as hay cutters (Lard et al. 2006), and to turf with the general upheaval of dirt caused by the creation of the fire ant's mound (Lard et al. 2006). In addition, approximately 1% of the population in the United States is highly allergic to fire ant venom and could potentially die when stung (Adams 1968). Fire ants are known to be highly aggressive and will defend their colony by biting and stinging

intruders when their mound is disturbed (Tshinkel 2006). This is a successful way for the ants to defend their colony against predators; however, this defensive mechanism can be quite painful and even deadly when humans are the seen as the unwelcome intruders. This makes fire ant control especially important in sensitive environments such as hospitals and schoolyards.

PSEUDACTEON PHORID FLIES

Imported fire ant populations in the United States are estimated to be five times greater than populations in their native South America (Porter et al. 1992; Porter et al. 1997). This is partially attributed to the lack of biological control agents in imported fire ant populations in the United States (Jouvenaz 1990; Porter et al. 1997). Several biological control agents are known to parasitize fire ants in South America. These include a protozoan, a couple of genera of nematodes, a fungus, several microsporidians, and multiple species of parasitoid flies.

Within the order Diptera, there are approximately 16,000 species in 21 families that are considered parasitoids (Eggleton & Belshaw 1992; Feener & Brown 1997). The family Phoridae contains members that are saprophagous, predators, or parasitoids (Brown 1992; Feener & Brown 1997). The members that are parasitoids are associated with social insects such as bees, ants and termites (Feener & Brown 1997). Species in the genus *Pseudacteon* are parasitoids of ants, including imported fire ants (Porter 1998).

Members of the genus *Pseudacteon* (Diptera: Phoridae) showed the most promise for successful release into Unites States fire ant populations as biological control agents (Porter 1998). These flies were extensively studied prior to introduction and were found

to only oviposit successfully on black imported fire ant and red imported fire ant populations; therefore, they pose little risk to native fire ant and other ant populations (Porter 2000). This factor makes them an excellent candidate for introduction, as they will likely not become an invasive pest themselves.

Scientists hypothesize that lower fire ant population densities, similar to populations in South America, can be attained in the United States with the introduction of biological controls (Porter et al. 1997). Over twenty species of *Pseudacteon* phorid flies have been identified attacking fire ants in South America (Taber 2000). Currently six species of *Pseudacteon* (*P. cultellatus, P. curvatus, P. litoralis, P. nocens, P. obtusus* and *P. tricuspis*) have been released into several fire ant infested states in the United States (Graham et al. 2001; Gilbert et al. 2008; Callcott et al. 2011; Porter et al. 2011; Plowes et al. 2012; Porter & Calcaterra 2013; Mészáros et al. 2014). In Alabama, five *Pseudacteon* spp. have been released (*P. cultellatus, P. curvatus, P. litoralis, P. obtusus* and *P. tricuspis*) (Fig. 1); however, only three species have been successfully established (*P. curvatus, P. litoralis,* and *P. tricuspis*) (Graham et al. 2003; Porter et al. 2011) (Fig. 2). For the purpose of this dissertation, only these three fly species will be addressed.

Pseudacteon spp. flies are often referred to as decapitating flies, due to the part their life cycle, when the host fire ant is decapitated. The phorid fly life cycle begins with the oviposition of an egg by the fly into the host ant's thorax, just behind the head (Wasmann 1918). The egg will hatch into a first instar larva and migrate into the fire ant's head. The exact time of migration into the head capsule is unknown. The second instar larva is found in the host's head capsule four days post injection (Porter et al. 1995b). Once inside the head capsule the fly larva will feed upon the host's hemolymph,

and the third instar will begin to cause the fire ant to make erratic motions (Henne & Johnson 2007). During this stage of development, the fire ant is often referred to as a "zombie ant," and the ant leaves the colony via lateral foraging tunnels just prior to its decapitation (Henne & Johnson 2007). The decapitation of the ant is caused by the release of chemicals by the third instar fly larva, which dissolves the intercuticular membrane between the fire ant's thorax and head capsule (Porter 1998). The developing fly larva will then push out the fire ant's mandibles, and the exposed segments of the larva will sclerotize to form a solid case with the remaining ant head capsule in which the fly will pupate (Morrison et al. 1997). Pupation takes two to six weeks. Overall development from injection of the egg to emergence of adult takes five to twelve weeks. Both are dependent upon the external temperature (Porter 1998). Adult flies emerge just before sunrise and are ready to mate and lay eggs just hours after emergence. Adult flies can lay up to 200 eggs and live one to three days in the field (three to seven days in a laboratory setting) (Porter 1998). Adult phorids have been found to live longer in a laboratory setting when provided with a sugar/nectar source (Chen et al. 2005; Fadamiro et al. 2005; Fadamiro & Chen 2005; Chen & Fadamiro 2006).

In their native South America, as many as eight species of *Pseudacteon* were found to inhabit the same geographic location (Porter et al. 1995a; Pesquero et al. 1996; Fowler 1997; Orr et al. 1997). *Pseudacteon curvatus*, *P. tricuspis*, and *P. litoralis* were among the species co-established in the same locations in South America (Pesquero et al. 1996). In order for these parasitoids to be successful, while using the same host ant species, it was found that each occupies a slightly different ecological niche (Porter 1998). An organism's place in the environment as a whole is known as its niche

(Schoener 2009). Each organism in the environment fits into a niche, and all factors in the ecosystem (availability of food, weather conditions, competition for resources, etc.) have impacts on this dynamic system. *Pseudacteon* spp. flies are closely related and have evolved unique ways in which they partition their resources to divide niche space, which decreases interspecific competition (Porter 1998).

First, different species of *Pseudacteon* flies are known to attack different size ranges of fire ant workers (Campiolo et al. 1994; Fowler 1997; Morrison et al. 1997). Fire ant workers are polymorphic and head widths range in size from approximately 0.45 mm-1.5 mm (Wood & Tschinkel 1981). *Pseudacteon curvatus* was shown to preferentially parasitize workers on the smaller end of the scale, those averaging 0.71 mm in head width (Morrison & Gilbert 1998). *Pseudacteon tricuspis* was shown to parasitize medium to large sized workers, those averaging approximately 0.92 mm in head width. The largest workers, those averaging 1.11 mm in head width, were parasitized by *P. litoralis*. All three of these species overlap slightly in their host size preference ranges (Sanford Porter, USDA-ARS, Gainesville, FL, personal communication). *Pseudacteon curvatus* and *P. tricuspis* host size preferences overlap at the upper end of the *P. curvatus* range and the lower end of the *P. tricuspis* range. *Pseudacteon tricuspis* and *P. litoralis* preferences overlap at the upper end of the *P. tricuspis* range and the lower end of the *P. litoralis* range.

Second, *Pseudacteon* spp. are known to attack fire ants that are engaged in different activities (Orr et al. 1997). *Pseudacteon curvatus* and *P. tricuspis* are only observed around disturbed fire ant mounds and fire ant mating flights (Smith 1928; Williams 1980; Pesquero et al. 1993; Bertagnolli & Graham 2005). These species were

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found to be most attracted to a combination of fire ant alarm pheromones and the venom alkaloids that the ants emit when their mound is disturbed (Chen & Fadamiro 2007; Chen et al. 2009; Chen & Fadamiro (I) 2009; Chen & Fadamiro (II) 2009; He & Fadamiro 2009; Chen et al. 2010; Sharma et al. 2011; Sharma et al. 2013). Studies have not been conducted into the exact attraction semiochemicals for *P. litoralis*. To date, this species has only been observed around disturbed fire ant mounds (Porter 1998). This suggests that similar host location cues to those used by *P. curvatus* and *P. tricuspis* are used by *P. litoralis*. Presence of *Pseudacteon* spp. around disturbed fire ant mounds decreases mound re-building efforts, causing a decline in overall colony health (Porter et al. 1995c). Other *Pseudacteon* spp. have been observed attacking fire ant foraging trails, which was shown to inhibit fire ant foraging ability during fly presence (Orr et al. 1997). These secondary impacts (impairment to mound rebuilding efforts/foraging efforts) were suggested to be the cause of lower fire ant population densities in South America compared to those observed in the United States (Porter et al. 1995c).

Finally, *Pseudacteon* spp. flies are known to attack fire ants at different times of the day and year (Pesquero et al. 1996). In Brazil, where *P. curvatus, P. tricuspis, P. litoralis* and other *Pseudacteon* species inhabit the same territory, *P. tricuspis* was observed to have one large peak in diurnal ovipositional activity that occurred mid-day (Pesquero et al. 1996). The same periods of activity were observed in the United States (Alabama and Florida) for *P. tricuspis* when it was independently established (Morrison et al. 1997; Bertagnolli & Graham 2004). *Pseudacteon tricuspis* was found to be active when temperatures were greater than 20 °C both in South America, as well as in Florida when independently established (Morrison & Porter 2005; Calcaterra et al. 2008).

Diurnal activity of *P. curvatus* was not documented in South America. In 2002, when *P. curvatus* was independently established in Alabama, it was shown to have two peaks in diurnal ovipositional activity that occurred during the middle of the day (Bertagnolli & Graham 2004). *Pseudacteon curvatus* was found to be active when temperatures were greater than 20 °C both in South America, as well as in Alabama when independently established (Bertagnolli & Graham 2004; Calcaterra et al. 2008). Both *P. curvatus* and *P. tricuspis* are found in the middle portion of the day, which provides an overlap in their diurnal activity patterns.

Pseudacteon litoralis was observed in Brazil to have two peaks in diurnal ovipositional activity, one occurring for a few hours post-sunrise and the other occurring for a few hours pre-sunset (Pesquero et al. 1996). This is a notably different diurnal cycle from both *P. curvatus* and *P. tricuspis*. *Pseudacteon litoralis* was found to be active when temperatures were greater than 20 °C in South America; however, it was found in the cooler part of the day (morning and late afternoon). This made *P. litoralis* less prevalent in the fall/winter months due to high temperatures (≥ 20 °C) only occurring in the middle portion of the day, which is opposite of their observed diurnal cycle (Fowler et al. 1995; Pesquero et al. 1996; Wuellner et al. 2002). Activity patterns in the United States were not observed for *P. litoralis* prior to the research that is detailed in this dissertation.

In South America, when multiple species of *Pseudacteon* are present, all sizes of ants are parasitized at all times of the day/year while different daily activities are performed for the ant colony. This creates a constant pressure upon the host fire ant colonies, which contributes to lower fire ant populations in South America than those observed in the United States (Porter 1998). In order for the fire ant biological program to

be successful in the United States, these same resource niches need to be filled. The research discussed in this dissertation will lend more insight into the interactions among the species once they are co-established in Alabama and will detail areas where future research is needed.

Four diurnal ovipositional studies were conducted to further the knowledge on how *P. curvatus, P. litoralis* and *P. tricuspis* coexist in Alabama. A seasonal abundance study was conducted in an area where *P. curvatus* and *P. tricuspis* are co-established in order to determine the best time of year to conduct studies on these species. Trapping methods for *P. litoralis* were tested with the goal of finding an easier and more time efficient way to monitor the spread of this species. Finally, the parasitism rates of *P. curvatus* and *P. tricuspis* were studied in order to compare these data with those obtained in South America where the species coexist, and Florida before the species were co-established.
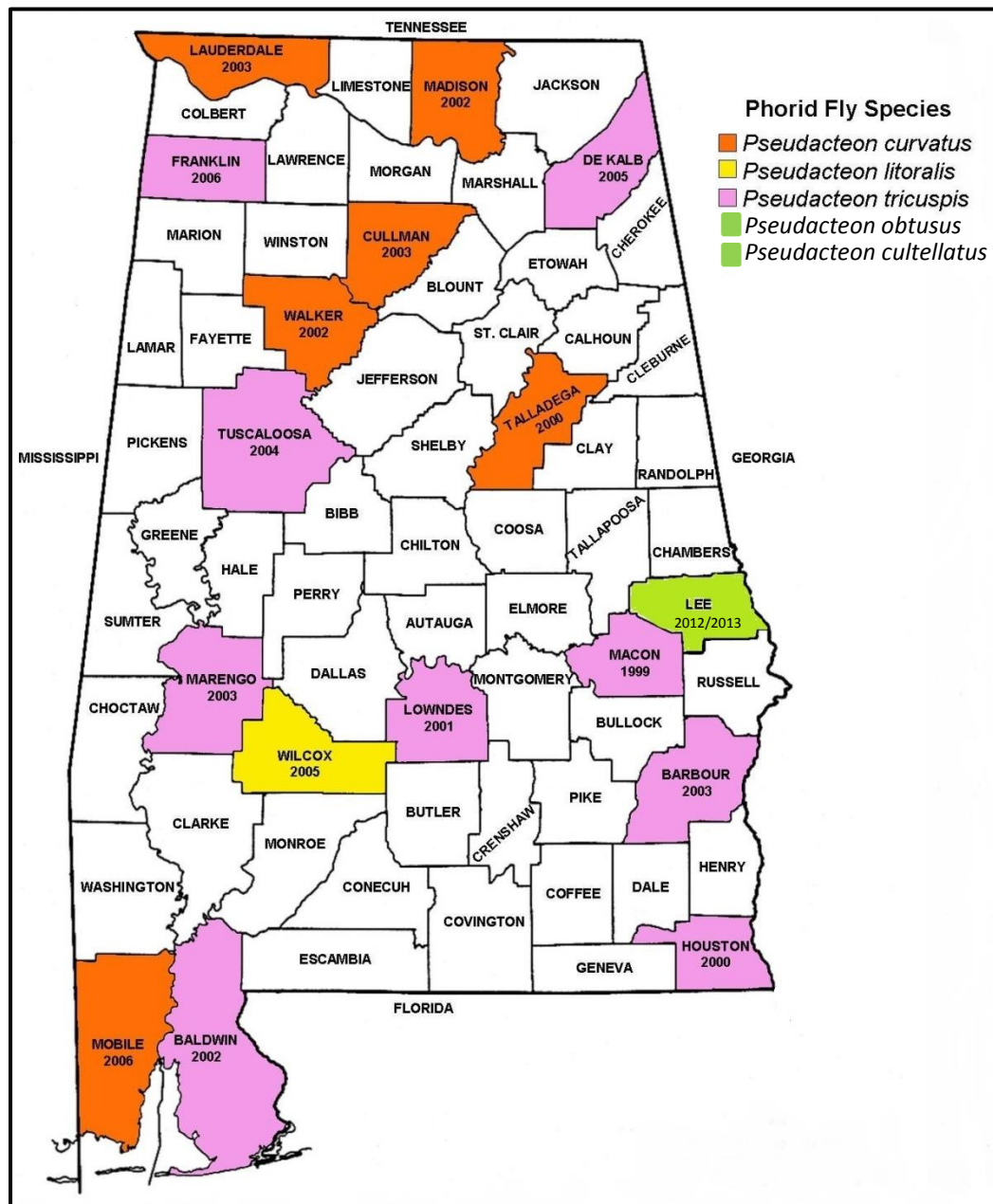
Fig. 1. *Pseudacteon* spp. release map of Alabama. Dates of releases appear under the county name.
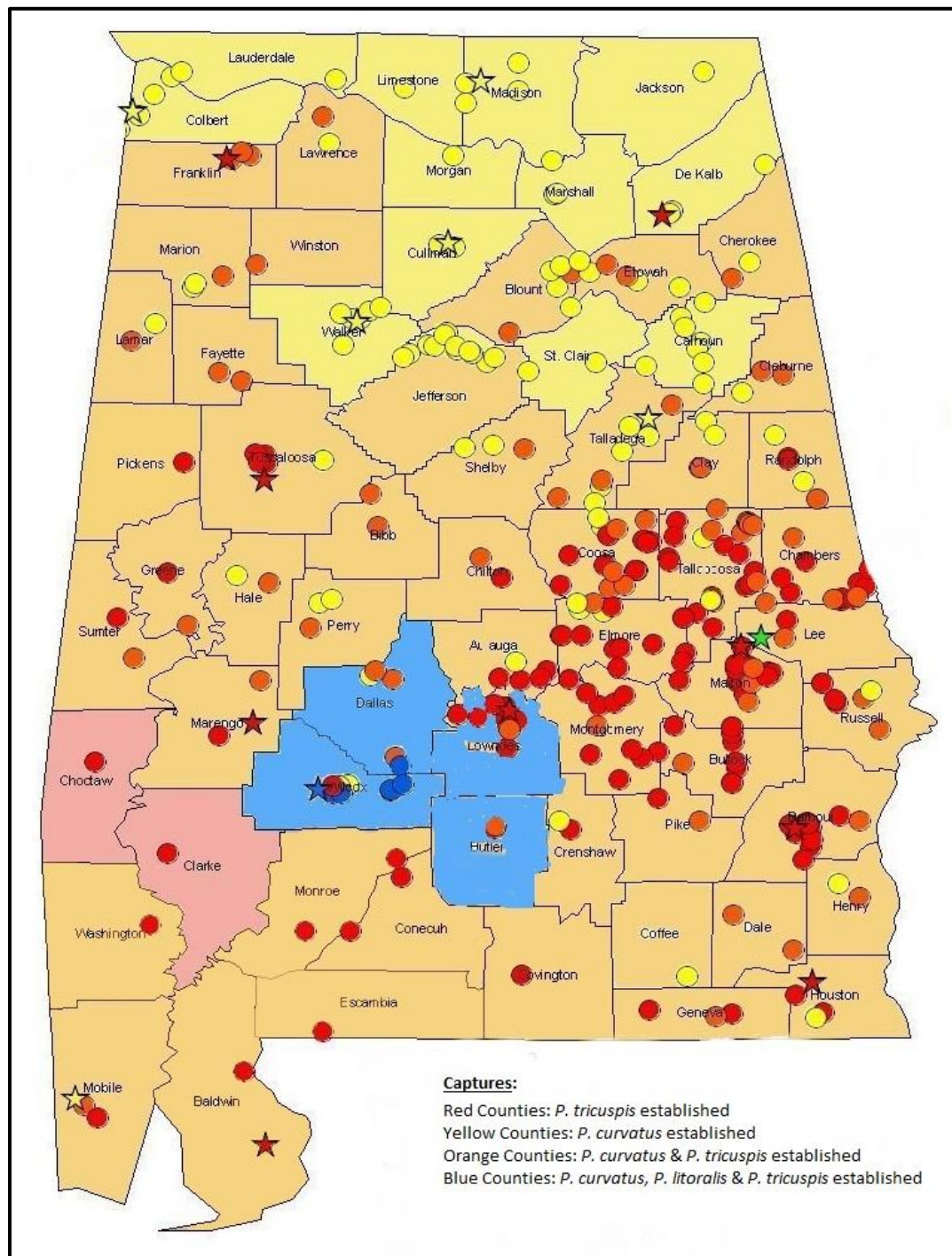
Fig. 2. 2014 Alabama *Pseudacteon* spp. distribution map. Red counties only have *P. tricuspis* established, yellow counties only have *P. curvatus* established, orange counties have both *P. curvatus* and *P. tricuspis* established, and blue counties have *P. curvatus, P. litoralis*, and *P. tricuspis* established.

II. Diurnal patterns of ovipositional activity of *Pseudacteon curvatus*, *Pseudacteon litoralis* and *Pseudacteon tricuspis* (Diptera: Phoridae) while co-established in Alabama

Background and Significance

In 1999 *Pseudacteon tricuspis* was released into Macon County, Alabama. This marked the first successful introduction of a parasitoid of imported fire ants in Alabama (Graham et al. 2001). This release was followed by the introduction of *Pseudacteon curvatus* into fire ant populations in Talladega County, Alabama in 2000. Releases of both *P. curvatus* and *P. tricuspis* continued, and by 2006 there had been six releases of *P. curvatus* into six counties in Alabama, and nine releases of *P. tricuspis* in nine counties. There was only one release of *Pseudacteon litoralis*, which took place in Wilcox County, Alabama in 2005 (Fig. 1).

By 2007, *P. curvatus* and *P. tricuspis* were co-established in 20 counties in Alabama, including Macon County and Talladega County, the first release sites. As of 2012, both *P. curvatus* and *P. tricuspis* inhabited 53 of 67 Alabama counties (Palmer & Graham, unpublished data). Today it is suspected that *P. curvatus* and *P. tricuspis* are co-established in all 67 Alabama counties. However, this cannot be confirmed due to limited funding for monitoring efforts. The only successful release of *P. litoralis* in the United States was in Alabama (Porter et al. 2011). *Pseudacteon curvatus, P. litoralis* and *P. tricuspis* are co-established in four Alabama counties (Fig. 2). *Pseudacteon litoralis* is also thought to have spread in range to additional counties; however, the extent is unknown.

In 2002, studies were conducted to determine the diurnal ovipositional patterns of *P. curvatus* and *P. tricuspis* in Alabama. At that time, each species occurred independently, with no overlap in geographical distribution. In 2009, by casual observation while collecting field data, I observed that numbers of *P. tricuspis* appeared to be tapering off when the species was co-established with *P. curvatus*. The same trend was observed in both Florida and Texas (Sanford Porter, USDA-APHIS, Gainesville, FL, personal communication; Robert Puckett, Texas A&M University, College Station, TX, personal communication).

Since the introduction of *P. curvatus, P. litoralis* and *P. tricuspis* into the United States, no data has been collected where all three species are co-established. This information is pivotal if we are to continue with releases of biological control agents for fire ants and may have important impacts on release protocols.

Four separate but related studies were conducted to determine the diurnal patterns of ovipositional activity of *P. curvatus, P. litoralis* and/or *P. tricuspis* while they were co-established in Alabama. The first study, conducted in 2010, observed the diurnal patterns of ovipositional activity of *P. curvatus* and *P. tricuspis,* when they were co-established. The second study, conducted between 2012 and 2013, repeated the previous 2010 study, providing another data set for comparison. The third study, conducted in 2010, observed the diurnal patterns of ovipositional activity of *P. curvatus, P. litoralis*, and *P. tricuspis* while they were co-established. The final study, conducted in 2013, repeated the third study to provide another data set for comparison.

I hypothesized that diurnal patterns of ovipositional activity would be different from those observed in South America due to an overlap in niche space (seasonal and

diurnal cycles) allowing for one species to be more successful when co-established with another phorid species. I also hypothesized that the diurnal patterns would be different from those observed in 2002, due to a presence or absence of interspecific competition that enables one species to be more successful than the other.

Materials and Methods

Study 1. Diurnal patterns of ovipositional activity where *Pseudacteon curvatus* and *Pseudacteon tricuspis* are co-established, Lee Co., 2010

This study was conducted between Jul and Sep 2010 at the farm of Phil and Daria Story (32°34'36.33"N, 85°35'42.58"W) in Lee County, Alabama. This study site is located approximately eight kilometers northeast of the original *P. tricuspis* release site in Macon County, Alabama (32°34'26.55"N, 85°39'55.03"W). Both *P. curvatus* and *P. tricuspis* have been established in this location since 2007.

Fire ants that were used in the study were collected outside of the study area, but within eight kilometers of the study location. This helped keep fire ant populations within the study location as consistent as possible throughout the study period. Mounds that measured at least 0.3 x 0.3 m were selected for excavation. Mounds of this size were used because it ensured that both: (1) a large amount of workers would be present and (2) representatives from many size classes would be present (Morrison & Porter 2005). Fire ants were excavated using a shovel and placed into a 5 gal bucket that was lined with Johnson's® baby powder which prevented ant escape. Two mounds were excavated for each sampling period. The buckets were transported to the lab where ants were separated from the soil by slowly dripping water from a plastic storage container (modified with a

valve to control water flow, 45 cm of latex tubing and a 1 mL pipet) into the bucket of ants placed below. This technique exploits the ability of the ants to survive floods in their native South America by making colony "rafts" and floating on the surface of the water until dry land is encountered (Jouvanez et al. 1977; Banks et al. 1981).

Forty grams of ants was determined to be the average weight of ants collected from a 0.3 x 0.3 m fire ant mound. This information was obtained by excavating five mounds measuring 0.3 x 0.3 m, separating the ants from the soil using the water drip method described above, and then averaging the weights of the ants collected. For each data collection 40 g of ants from each of two fire ant colonies were weighed and placed into separate and labeled 52 x 40 x 13 cm trays. The top edge of the trays were lined with Fluon® (Asahi Glass LTD, Chadds Ford, PA) to prevent the ants from escaping.

The ants were supplied with a 4 g slice of Gwaltney® chicken hot dog, and two 15 x 95 mm plastic test tubes, one filled with water, and the other with a 20% sugar water solution. Each test tube had a cotton ball stuffed in the end to allow the ants to access the contents of the tubes, while preventing the liquids from spilling. Ants were also provided with a nest cell that consisted of a 150 x 15 mm Petri dish, which was filled with dental plaster that was mixed and cured according to the label directions. Once cured, the nest cell was held under water until saturated, excess moisture was dried off, and the cell was placed into the tray. The nest cell provided the ants with moisture which prevented desiccation. The ants were kept in the lab no longer than 1 d after collection before the studies were conducted, ensuring the ants would not acclimate to the laboratory setting. New colonies of ants were obtained in this manner for each data collection period.

The trays of ants were transported to the field site and placed 100 m apart in shady locations that were protected from wind movement. Phorids have been found to be attracted to host fire ants at distances of less than 50 m (Morrison et al. 1999). Therefore, trays were placed 100 m apart so as not to get competition between boxes for phorid attraction (Sanford Porter, USDA-APHIS, Gainesville, FL, personal communication). Ants were placed in the shade, which was cooler than areas in the sun, to ensure that the ants stayed active during data collection. Ants were also placed in areas surrounded by trees, which limited wind movement. This was important because phorid flies are quite small and can be easily manipulated by the wind. Tray locations were marked using flags that were labeled with either "tray 1" or "tray 2." Flags were left in the field for the entire study period, and each tray was placed in the same location for each data collection period.

In order to attract phorid flies the trays of ants were shaken to agitate the ants, which made them release both the alarm pheromone and venom alkaloids. Trays were shaken at sunrise on each collection day starting with tray 1 and followed immediately by tray 2. Thirty minutes after shaking, flies that were around tray 1 were aspirated using a double-chambered aspirator unit® (BioQuip Products Inc., Rancho Dominguez, CA, USA). This aspirator was used because it allowed for live collection of flies into a 2-dram vial, making data collection faster and easier. Flies were collected until no flies were observed around the tray. Then, tray 1 was shaken again to agitate the ants, and the collection and agitation process was repeated for tray 2. Separate and labeled aspirators were used for each tray to ensure that data were appropriately recorded.

Once the flies were collected from each tray, they were transferred to separate and labeled 14 x 14 x 7 cm plastic Rubbermaid® containers via a small hole in the container lid. Transferring the flies into a larger container made identification of flies easier and faster. Gaseous CO_2 was introduced into the plastic container via the small hole in the lid to anesthetize the flies. Once flies were knocked down they were identified to species using a 10x hand lens and sexed. The lids of the plastic containers were then removed and the containers placed into shady locations near the trays. This allowed for fly recovery and release, which ensured more accurate sampling during the diurnal activity study since the local population, was not reduced by fly removal.

This entire process was repeated every 30 min from sunrise until sunset for each data collection period. In order for data to be consistent hours after sunrise was used instead of the actual time of day. Data were collected two times per week, two times per month, for the three month study period. Due to time constraints, data sets were split over a two day period, each beginning 6.5 h after sunrise until sunset, and continuing the next day from sunrise until 6 h after sunrise. The collection dates were as follows: 21 and 22 Jul 2010; 23 and 24 Jul 2010; 26 and 27 Jul 2010; 28 and 29 Jul 2010; 3 and 4 Aug 2010; 9 and 10 Aug 2010; 12 and 13 Aug 2010; and 14 and 15 Aug 2010. One full-day of data were collected on 7 Sep 2010, and this was the final data set for this study. This provided nine complete data sets for this study.

Study 2. Diurnal patterns of ovipositional activity where *Pseudacteon curvatus* and *Pseudacteon tricuspis* are co-established, Lee Co., 2012-2013

The 2010 diurnal ovipositional activity study was repeated between Jun 26, 2012 and Jun 12, 2013. The materials and methods used for this study are the same as those described above, as was the study location, however, this study collected data over a longer observation period. Data were collected two times per week and two times per month for an entire calendar year. Also, in this study, the collection periods were not split over two days. Instead, entire data sets were collected from 0.5 h after sunrise until sunset of that same day. Data collection periods were as follows: 26 and 28 Jun 2012; 3, 4, 17 and 19 Jul 2012; 2, 3, 15 and 16 Aug 2012; 6, 7, 24 and 26 Sep 2012; 8, 10, 21 and 29 Oct 2012; 19, 20, 26 and 28 Nov 2012; 3, 4, 13 and 14 Dec 2012; 18, 19, 26 and 27 Jan 2013; 15, 16, 23 and 24 Feb 2013; 22, 24, 29 and 30 Mar 2013; 6, 7, 27 and 28 Apr 2013; 8, 10, 30 and 31 May 2013; and 10 and 12 Jun 2013.

Study 3. Diurnal patterns of ovipositional activity where *Pseudacteon curvatus*, *Pseudacteon litoralis* and *Pseudacteon tricuspis* are co-established, Wilcox Co., 2010

The third diurnal ovipositional activity study was conducted at the Biddle farm (31°58'29.05"N, 87°04'22.88"W), which was the original release site for *P. litoralis* in Wilcox County, Alabama. *Pseudacteon curvatus, P. litoralis* and *P. tricuspis* have been co-established in this location since 2007. The materials and methods are the same as described in the first objective, however, there were only a total of five complete data sets. This site is located 2.5 h from our Lee County, Alabama office, which made it difficult to get to the site. Therefore, the data sets were split over a two day period, each

beginning 6.5 h after sunrise until sunset, and continuing the next day from sunrise until 6

h after sunrise. The data collection periods were as follows: 29 and 30 Jul 2010; 5 and 6

Aug 2010; 1 and 2 Sep 2010; 15 and 16 Sep 2010; and 29 and 30 Sep 2010.

Study 4. Diurnal patterns of ovipositional activity where *Pseudacteon curvatus, Pseudacteon litoralis* and *Pseudacteon tricuspis* are co-established, Wilcox Co., 2013

The 2010 study on diurnal ovipositional activity was repeated between Jul 31 and

Oct 2 of 2013. Due to complications at the original release site of *P. litoralis*, this study

was conducted at the farm of Walt and Peggy Prevatt (32°01'03.36 N, 87° 00'30.95 W)

in Wilcox County, Alabama. This site was approximately ten kilometers from the original

P. litoralis release site. The same materials and methods were used as in the first study,

however, due to the travel time, data sets were split between two days. The data were

collected starting at 6.5 h after sunrise and continued until sunset. Then, continued the

next day from sunrise until 6 h after sunrise. Ten total data sets were collected. The data

collection periods were as follows: 31 Jul and 1 Aug 2013; 13 and 14 Aug 2013; 14 and

15 Aug 2013; 20 and 21 Aug 2013; 27 and 28 Aug 2013; 3 and 4 Sep 2013; 11 and 12

Sep 2013; 16 and 17 Sep 2013; 23 and 24 Sep 2013; and 1 and 2 Oct 2013.

Results

Study 1. Diurnal patterns of ovipositional activity where *Pseudacteon curvatus* and *Pseudacteon tricuspis* are co-established, Lee Co., 2010

There were two peaks in ovipositional activity for *P. curvatus* (Fig. 3). The first occurred between 3.5 h and 5.5 h after sunrise and averaged 8.5 flies per collection period. The second peak was slightly smaller and occurred between 10.5 h and 12.5 h after sunrise and averaged 7.3 flies per collection period. *Pseudacteon curvatus* was consistently present throughout the middle part of the day (between 6 h and 10 h after sunrise) and averaged 4.6 flies per collection period (raw data in Appendix 1).

The activity patterns of *P. tricuspis* were sporadic, occurring primarily in the middle part of the day between 2.5 h and 11.5 h after sunrise. All collections of *P. tricuspis* averaged less than one fly (0.22 flies) per collection period.

Study 2. Diurnal patterns of ovipositional activity where *Pseudacteon curvatus* and *Pseudacteon tricuspis* are co-established, Lee Co., 2012-2013

There were two peaks in ovipositional activity for *P. curvatus*. The highest peak occurred between 4 h and 4.5 h after sunrise (Fig. 4). This peak in activity averaged 9.8 flies per collection period. The second, smaller peak occurred between 11 h and 12.5 h after sunrise and averaged 6.2 flies per collection period. During the middle part of the day, between 5.5 h and 10.5 h after sunrise, *P. curvatus* was consistently present, and numbers averaged 3.6 flies per collection period (raw data in Appendix 2).

Few *P. tricuspis* were collected during this study. Flies were collected in the middle part of the day between 3 h and 10.5 h after sunrise. An average of 0.007 flies were collected.

Study 3. Diurnal patterns of ovipositional activity where *Pseudacteon curvatus, Pseudacteon litoralis* and *Pseudacteon tricuspis* are co-established, Wilcox Co., 2010

Diurnal activity of *P. curvatus* had only one peak of activity, which occurred in the afternoon between 10 h and 12 h after sunrise (Fig. 5). This peak averaged 13.3 flies over the collection periods. *Pseudacteon curvatus* was collected consistently between 2 h and 9.5 h after sunrise and averaged 5.0 flies over those collection periods (raw data in Appendix 3).

Pseudacteon litoralis had two peaks of activity. The highest peak in activity occurred between 1.5 h and 3.5 h after sunrise. This peak in activity averaged 21.3 flies per collection period. The second peak in diurnal activity was much smaller and occurred in the afternoon between 12.5 h and 13.5 h after sunrise. This peak in activity averaged 1.9 flies per collection period. *Pseudacteon litoralis* was not collected between 5 h and 12 h after sunrise.

Pseudacteon tricuspis was consistently present between 1.5 h and 13 h after sunrise. Activity averaged 3.1 flies per collection period.

Study 4. Diurnal patterns of ovipositional activity where *Pseudacteon curvatus, P. litoralis* and *Pseudacteon tricuspis* are co-established, Wilcox Co., 2013

There was one large peak in diurnal activity for *P. curvatus* that occurred between 6.5 h and 8.5 h after sunrise (Fig. 6). Activity averaged 34.7 flies per collection period during this peak. *Pseudacteon curvatus* was present between 1.5 h and 13.5 h after sunrise, and averaged 15.3 flies over these collection periods (raw data in Appendix 4).

Pseudacteon litoralis had two peaks in diurnal activity, the highest peak occurred in the morning between 1.5 h and 3.5 h after sunrise. This peak averaged 13.8 flies per collection period. The second, smaller peak in activity occurred in the late afternoon between 12 h and 13 h after sunrise. The peak averaged 4.7 flies per collection period. *Pseudacteon litoralis* was not present between 6 h and 9 h after sunrise.

Psuedacteon tricuspis was present between 2 h and 11 h after sunrise. Diurnal activity averaged 1.4 flies per collection period.
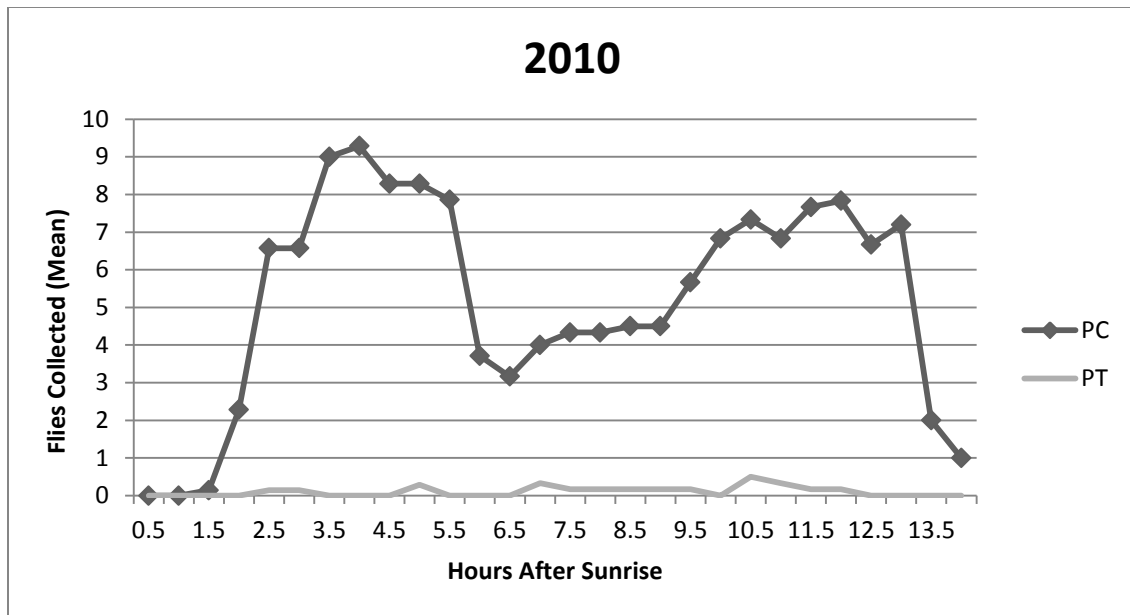
Fig. 3. Diurnal patterns of ovipositional activity when *Pseudacteon curvatus* (PC) and *P. tricuspis* (PT) are co-established, Lee Co., 2010.
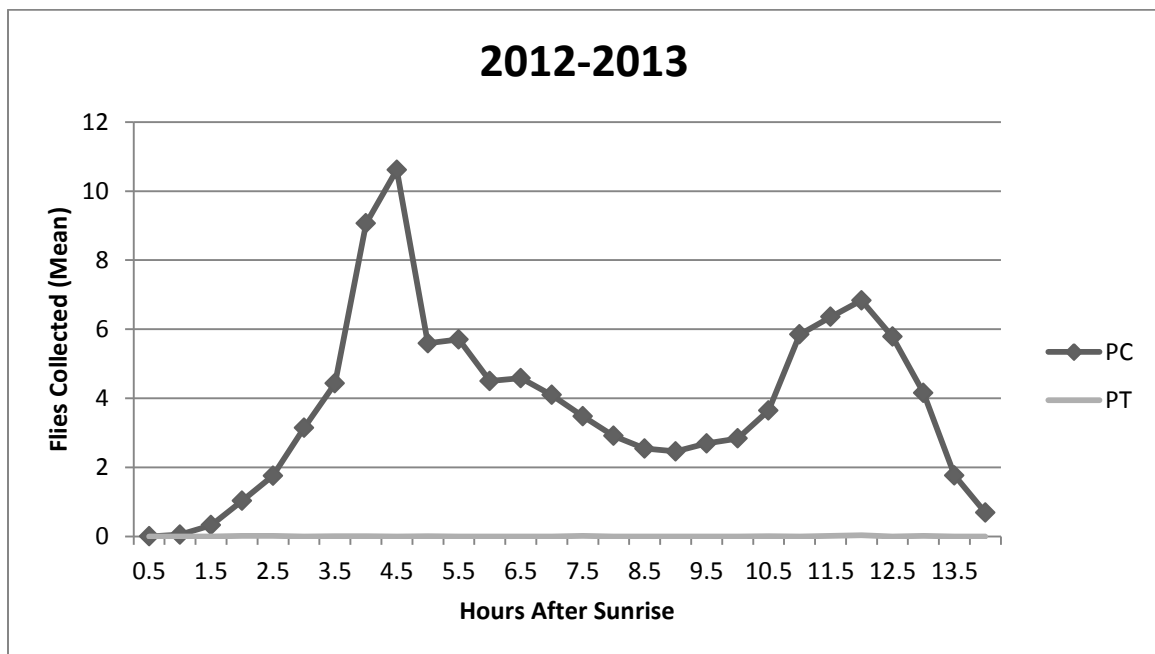
Fig. 4. Diurnal patterns of ovipositional activity where *Pseudacteon curvatus* (PC) and *P. tricuspis* (PT) are co-established, Lee Co., 2012-2013.

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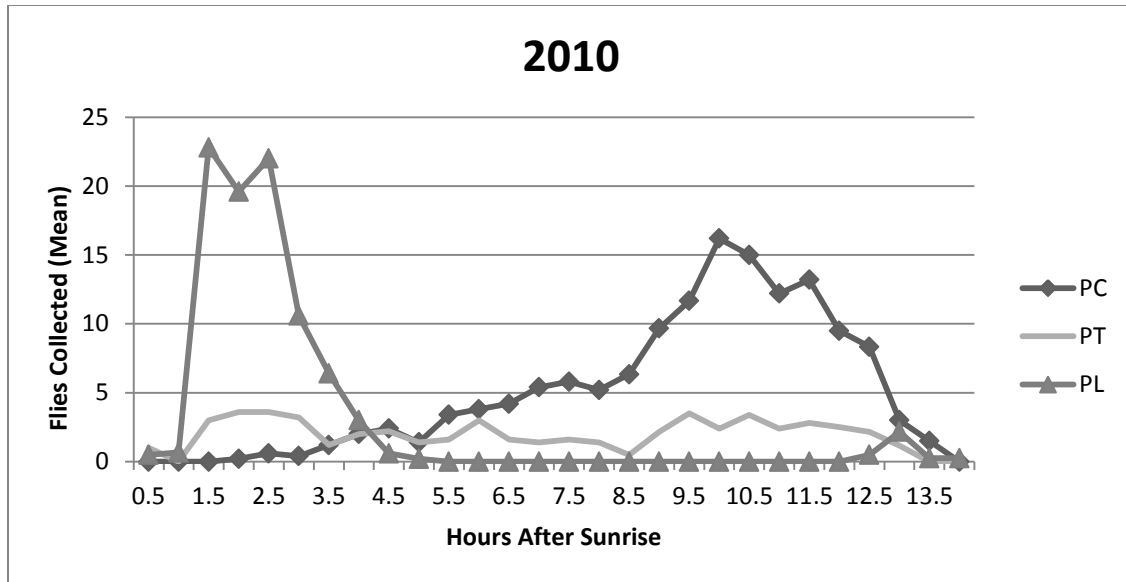
Fig. 5. Diurnal patterns of ovipositional activity where *Pseudacteon curvatus* (PC)*, P. litoralis* (PL) and *P. tricuspis* (PT) are all co-established, Wilcox Co., 2010.
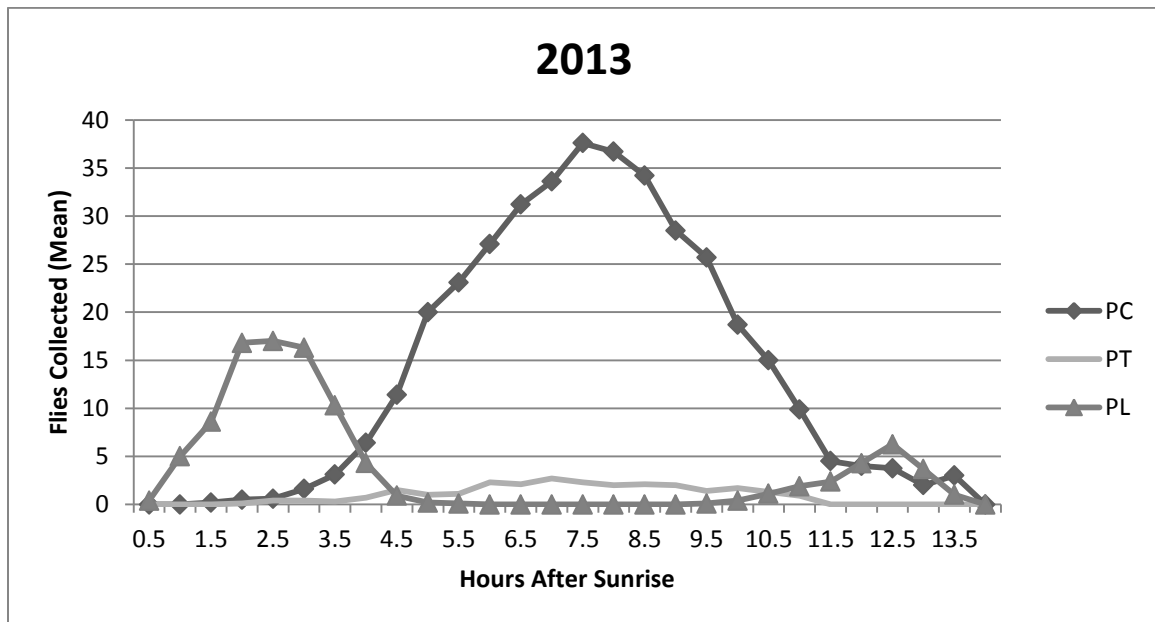
Fig. 6. Diurnal patterns of ovipositional activity where *Pseudacteon curvatus* (PC)*, P. litoralis* (PL) and *P. tricuspis* (PT) are co-established, Wilcox Co., 2013.

Discussion

In 2002, when *P. curvatus* was independently established, it was found to be active between 1.5 h and 13 h after sunrise, and had two peaks in ovipositional activity (Bertagnolli & Graham 2004; Bertagnolli & Graham 2005). The first peak in activity was smaller and occurred at 4.5 h after sunrise; there was a larger peak in the afternoon, occurring at 10.5 h after sunrise. *Pseudacteon curvatus* was consistently collected throughout the middle portion of the day during this study. During their peak abundance period, *P. curvatus* numbers averaged 65 flies per collection period (Bertagnolli & Graham 2004; Bertagnolli & Graham 2005). *Pseudacteon curvatus* diurnal ovipositional activity patterns were not observed in South America, therefore, the 2002 data collected in Alabama serves as a baseline for comparison.

Data collected in the 2010 and 2012-2013 studies above suggest a shift in the ovipositional patterns of *P. curvatus* when it is co-established with *P. tricuspis*. *Pseudacteon curvatus* is still present in the field consistently throughout the middle portion of the day, but the latest data suggest that the ovipositional peaks in mid-morning and afternoon are of similar size. Since there was no data collected on the diurnal ovipositional patterns of *P. curvatus* while it is co-established with other *Pseudacteon* spp. in South America, it is unknown if this pattern of abundance is unique to Alabama. There was a vast difference in the size of the data sets between the 2002 data and the 2010 / 2012-2013 data sets, which could attribute to the differences in activity observed.

When *P. curvatus, P. litoralis* and *P. tricuspis* were co-established, *P. curvatus* activity patterns shifted and only showed one large peak between 6.5 h and 8.5 h after sunrise, while being collected consistently throughout the middle portion of the day. This

was observed in both the 2010 as well as the 2013 data sets. In the 2010 data, the peak was observed later in the day than was observed in the 2013 study. This is the first data that has ever been collected when these three species are co-established. There was no baseline data from South America on *P. curvatus*, therefore we can't say whether this activity pattern is unique to Alabama.

Pseudacteon litoralis was determined in South America to be active in the early morning (2.5 h to 3.5 h after sunrise) and late afternoon (10.5 h to 12.5 h after sunrise) with little to no activity observed in the middle portion of the day (Pesquero et al. 1996). This pattern held true in Alabama in the 2010 and 2013 studies. *Pseudacteon litoralis* has a different diurnal activity pattern from both *P. curvatus* and *P. tricuspis*. There is little overlap in the activity patterns of *P. litoralis* and *P. curvatus*/*P. tricuspis*, which probably allows this species to be successful without much interaction with the other two phorid species.

Perhaps the most interesting and intriguing observations were the diurnal patterns of activity (or lack thereof) of *P. tricuspis* in the 2010 and 2012-2013 studies. In 2002, when *P. curvatus* and *P. tricuspis* were both independently established in Alabama, both species were found in equally high abundance in their respective locations (Bertagnolli & Graham 2004). *Pseudacteon tricuspis* was found to occur throughout most of the day in the 2002 and showed one large peak in activity that occurred around 6 h after sunrise (Bertagnolli & Graham 2004; Bertagnolli & Graham 2005). During this peak in activity, *P. tricuspis* numbers averaged 60 flies per collection period (Bertagnolli & Graham 2004; Bertagnolli & Graham 2005). A very similar trend was observed in South America, where *P. tricuspis* was active most of the day and had a peak in activity around 7 h after

sunrise (Pesquero et al. 1996). In the most recent studies, *P. tricuspis* numbers are much

lower (< 1 fly per collection period) when co-established with *P. curvatus*. This same

trend was observed when *P. tricuspis* was co-established with *P. curvatus* and *P.*

litoralis.

There are many possible hypotheses as to why this trend is occurring. In South

America as many as eight *Pseudacteon* spp. have been collected around the same fire ant

mound. These species evolved together and are able to maintain relatively equal

population numbers due to successful division of niche space, which reduces interspecific

competition (Calcaterra et al. 2008). One hypothesis is that *P. curvatus* is able to out-

compete *P. tricuspis* for resources when co-established in the United States. When

Pseudacteon spp. were introduced into the United States, they were individually released

and populations grew without any interaction with other phorid species for several years.

Both *P. curvatus* and *P. tricuspis* have diurnal activity patterns that occupy the middle

portion of the day. This overlap in diurnal cycle could allow *P. curvatus* to out-compete

P. tricuspis for resources.

Difference in diurnal cycle could be the reason that *P. litoralis* is able to be

successful when established with *P. curvatus* and *P. tricuspis*. *Pseudacteon litoralis*

occupies the early morning and late afternoon hours. This provides little interaction

between *P. litoralis* and the other two phorid species. This is apparent by the high

population numbers and activity patterns that are unchanged from those observed in

South America.

This hypothesis is supported by one observance in South America where it was

noted that *P. curvatus* was absent from data collections. During these collections, another

phorid species (*P. cultellatus*), which occupies the same diurnal cycle and prefers the smallest fire ant workers (0.51 mm mean head width), was thought to be out-competing *P. curvatus* for resources (Calcaterra et al. 2008).

Interspecific competition has been noted as a potential reason for the inability to get other *Pseudacteon* species established in the U.S. *Pseudacteon litoralis* was only established in one (Alabama) of the nine total releases that included sites in Alabama (one site), Florida (six sites), Louisiana (one site), and Mississippi (one site) (Porter et al. 2011). Competition was listed as a potential factor limiting the establishment of *P. litoralis*, but could not be determined to be the only factor involved. In Texas, the establishment of *P. obtusus* was found to be 35 times more likely when other *Pseudacteon* species were not already established in the area of release, and it was suggested that interspecific competition was a potential reason for this difference in success rate (Plowes et al. 2011). Competition between species was again listed as a potential reason for the apparent displacement of *P. tricuspis* by *P. curvatus* in Texas; however, it could not be sufficiently defended as the primary cause (Lebrun et al. 2009).

Historically, the hypothesis of competition for hosts between biological control agents and its influence on successful pest reduction is controversial. The original approach to biological control of a non-native species was to release as many biological control agents as possible, with the hopes that one will successfully establish and control the pest species (Myers 1985; Myers et al. 1989; Denoth et al. 2002). However, it was noted that when multiple species were released for pest suppression, typically only one of the biological control agents was responsible for the control of the pest (Myers 1985; Myers et al. 1989). In 56% of the successful biological control release projects studied by

Denoth and others (2002) credited a single species for the overall success. The theory of competitive exclusion was proposed as one of the detriments of release of multiple species for biological control of a pest (Ehler & Hall 1982). Meaning, that the competition between the biological control agents for hosts could ultimately eliminate many of the agents released, thus leaving only one agent successfully established. Competitive exclusion was supported with the evidence that significantly more biological control successes were attributed to single species releases compared to multiple agent releases (Ehler & Hall 1982).

Even though competition between species was given much attention as the major factor influencing biological control establishment success, there were also many critics of this theory. The major factor that spurred the opposition was the lack of other alternative explanations that could allow for the success of one biological control agent over another (Keller 1984). One cannot say that only one factor (competition) is the sole reason for the success of one species over another in an environment; therefore, multiple factors need to be considered. Also, it is suggested that more time should be taken to determine the best biological control agent for release rather than introducing multiple species in a "lottery" release (Myers et al. 1989).

The differences in the success rates among *Pseudacteon* species should also be subject to the objectivity described above. Competition between species cannot be ruled out as a cause of success of *P. curvatus* over *P. tricuspis*, and it cannot be the only reason proposed as the reason for this success. Another hypothesis for the success of *P. curvatus* over *P. tricuspis* is that *P. curvatus* simply has more host fire ants in the environment to parasitize. *Pseudacteon curvatus* prefers to oviposit fire ants that are small to medium in

size, those falling into the minor size class (0.71 mm in head width), in relation to the

other fire ant workers (Morrison & Gilbert 1998). *Pseudacteon tricuspis* prefers to

oviposit in medium to larger fire ant workers, those falling into the major size class (0.92

mm in head width). Fire ant worker sizes are greatly influenced by the age of the colony.

Younger fire ant colonies (< 1 yr old) tend to have significantly more minimum (< 0.5

mg in weight, 0.51 mm in head width) and minor (0.5-2.0 mg in weight, 0.55-0.75 mm in

head width) workers (Markin et al. 1966; Tschinkel 1988). As the colony matures, more

minor workers are present than any other caste (Tschinkel 1988). This is important

because overall there are a greater number of minimum and minor workers present in the

environment, which gives *P. curvatus* an advantage over *P. tricuspis* simply by having

more host workers.

Faster developmental time is another potential hypothesis for the success of *P.

curvatus* when co-established with *P. tricuspis*. The developmental time for *P. curvatus*

(both males and females) is shorter than for *P. tricuspis* due to the size difference of the

flies (Folgarait et al. 2002). *Pseudacteon curvatus* developmental time averaged 31 days,

whereas *P. tricuspis* developmental time averaged 38 days in a laboratory setting

(Folgarait et al. 2002). This could contribute to the success of *P. curvatus*, and could

significantly increase their population numbers to the point where they are overwhelming

P. tricuspis at ovipositional sites.

Another hypothesis is that *P. curvatus* is better able to adapt to climatic changes

in the United States than *P. tricuspis*. In South America, both *P. curvatus* and *P. tricuspis*

were found ovipositing throughout the year when temperatures were greater than 20 °C

(Calcaterra et al. 2008). At the sites where both species are established, the temperatures

rarely were observed to be less than 20 °C in the middle part of the day. However, in

Alabama temperatures are much lower in the winter months, providing many more days

with maximum temperatures below the 20 °C temperature threshold (Alabama Weather

Information Services 2014). *Pseudacteon curvatus* could simply be better equipped to

handle the cooler winter temperatures found in Alabama, thus making it more successful

than *P. tricuspis*.

When fire ants were observed in a laboratory setting, they displayed several

behavioral responses to the presence of phorid flies (Wuellner et al. 2002). Fire ants were

shown to freeze in place and huddle together for protection, others were shown to raise

their abdomen in a defensive stance, and some would curl up into a C-shaped stance for

protection. *Pseudacteon curvatus* and *P. tricuspis* were among the phorid species studied

for their influence on fire ant behaviors. *P. curvatus* had the least impact on fire ant

behaviors, whereas *P. tricuspis* had a greater impact on fire ant behavioral changes. This

difference could allow for *P. curvatus* to have more chances for oviposition than *P.

tricuspis*, which provides another hypothesis that could attribute to their overall success.

The trend for the decrease in *P. tricuspis* numbers when it is co-established with

P. curvatus has been noted in both Florida (Sanford Porter, USDA-APHIS, Gainesville,

FL, personal communication) and Texas (Robert Puckett, Texas A&M University,

College Station, TX, personal communication). However, data on abundance or diurnal

activity has not been formally documented to show these differences.

These findings are important for the future of the fire ant biological control

program. When *Pseudacteon* spp. were initially introduced into the United States as

biological control agents, it was noted that multiple species would be needed in order for

the program to be successful (Pesquero et al. 1996). This statement is very true; however, these studies indicate that it may be necessary to release multiple species together into new locations. This is especially true when the species occupy the same diurnal pattern, preferentially oviposit on the same size fire ant workers and/or are attracted to the same host semiochemicals. If species are released together, it could be more difficult for one species to attain overwhelming population numbers, and thus, the program could be more successful. Also, this data may show that it would be pointless to continue to release additional *Pseudacteon* spp. that exploit the same host preferences as *P. curvatus* into areas where *P. curvatus* is already established. Additional research is needed into the exact cause of the decline in *P. tricuspis* numbers while it co-exists with other *Pseudacteon* spp. This research will be pivotal for the future of the fire ant biological control program in the United States.

III. Seasonal abundance of *Pseudacteon curvatus* and *Pseudacteon tricuspis* (Diptera: Phoridae), while co-established, in Alabama

Background and Significance

As of 2012, *P. curvatus* and *P. tricuspis* were co-established in 53 of 67 Alabama counties (Palmer & Graham, unpublished data). However, it is believed that these species are present in all 67 Alabama counties, but due to limited funding for monitoring this is unknown. As these species continue to spread into new locations and new species of *Pseudacteon* phorid flies are introduced, it is important to understand how the species are interacting once co-established. This includes seasonal abundance patterns. With this information, researchers can target the periods when phorids are most abundant, and this will make future studies involving these flies much easier.

The seasonal abundance of *P. tricuspis* has been determined when it is co-established with other *Pseudacteon* spp. in South America (Pesquero et al. 1996; Calcaterra et al. 2008). These patterns have also been documented when it was independently established in Alabama and Florida (Bertagnolli & Graham 2004; Morrison & Porter 2005). However, the seasonal abundance of *P. tricuspis* has not been determined when it is co-established with other *Pseudacteon* spp. in the United States.

Pseudacteon curvatus seasonal abundance patterns were not determined in South America. Though it was found co-established with other phorid species in South America, its abundance was sporadic, and thus these data were not analyzed. Seasonal abundance of *P. curvatus* when it was independently established was observed in Alabama in 2002 (Bertagnolli & Graham 2004; Bertagnolli & Graham 2005).

Where multiple *Pseudacteon* spp. are established in South America ambient temperatures rarely fall below 20 °C (Calcaterra et al. 2008). This temperature was found to be the threshold for *Pseudacteon* spp. flight. When temperatures fell below 20 °C flight activity ceased. In Alabama, temperatures often fall below this threshold, especially between the months of October and April (Alabama Weather Information Services 2014).

I hypothesized that the seasonal abundance of *P. tricuspis* will be different from the patterns observed in South America due to differences in annual temperatures. I also hypothesized that the seasonal abundance patterns of both *P. curvatus* and *P. tricuspis* will be altered from abundance patterns observed while they were independently established. This will be due to an overlap in niche space which allows for one species to be more successful than another with two species are co-established.

A year-long study was conducted in Alabama to determine the patterns of seasonal abundance of *P. curvatus* and *P. tricuspis* while co-established.

Materials and Methods

The study was conducted just behind our research lab in Lee County, Alabama (33°35'57.28"N, 85°30'03.33"W). Both *P. curvatus* and *P. tricuspis* have been established since 2007. In order to monitor for seasonal abundance, traps were used to collect phorids from the field. These traps were developed by Robert Puckett, a research scientist with Texas A&M University, in 2007, and were found to be highly efficient in collecting both *P. curvatus* and *P. tricuspis* (Puckett et al. 2007). Traps consisted of a 100 x 15 mm Petri dish that was connected to the inside of a 150 x 15 mm petri dish using a

hot glue gun. Then, a Dixie Pizza Tri-Stand® (PTS) was hot glued in the middle of the interior Petri dish (Fig. 7). The PTS was then coated with Tanglefoot Insect Trap Coating® (Tanglefoot Co., Grand Rapids, MI) using a wooden stick applicator.

Two grams of freshly frozen fire ants were randomly collected, weighed and placed into the interior Petri dish before the traps were placed in the field. The frozen ants served as the phorid attractant (Puckett et al. 2007). Puckett et al. (2007) proved that this is as effective at monitoring abundance as aspirating phorids from around a disturbed mound. Fire ants used as attractants in the study were obtained outside of the collection area, but within approximately eight kilometers of the study site. This helped keep local fire ant population densities as constant as possible within the study area. Fire ant mounds that measured 0.3 x 0.3 m were excavated using a shovel and placed into five gallon buckets lined with Johnson's® Baby Powder, which prevented ant escape. Mounds of this size were used because it ensured that both: (1) a large amount of workers would be present and (2) representatives from many size classes would be present (Morrison & Porter 2005). Fire ants were separated from the soil using the same water drip technique that was described in the first objective.

Once the fire ants were separated from the soil they were transferred into a 52 x 40 x 13 cm tray using a slotted plastic spoon. The tray was lined with Fluon® (Asahi Glass LTD., Chadds Ford, PA) which prevented ant escape. Ants separated from the soil in the morning were left out in the lab in their trays until late afternoon to allow for any moisture that was transferred from the bucket to dry. If too much moisture remained from the bucket of water, the ants would freeze to the bottom of the tray where they couldn't be used. Once ants were dry, the tray was placed into the freezer overnight to kill the

ants. New mounds were excavated, separated from soil, and frozen for each trapping date.

Ten trapping locations were selected; each located was no less than 100 m from any other trap. Phorids have been found to be attracted to host fire ants at distances of less than 50 m (Morrison et al. 1999). Therefore, traps were placed 100 m apart so as to avoid competition between them (Sanford Porter, USDA-APHIS, Gainesville, FL, personal communication). Locations were marked with flags. The locations were also marked using a hand held GPS device to allow us to locate the trapping locations should something happen to the flag markers. Traps were labeled according to trap location and date. Ten traps were placed into the field on each sampling day. Traps were placed in the field on Mondays, Wednesdays and Fridays of each week and left out for 24 h. Therefore, traps were collected on Tuesdays, Thursdays and Saturdays at the same time they were placed out the previous day. This was continued every week for one year, beginning Jun 18, 2012 and ending Jun 13, 2013. Once traps were collected from the field, all phorids present on the traps were identified to species, sexed, and the data recorded. Traps were reused throughout the study and were cleaned using only water and paper towels to prevent any other fragrances from interfering with phorid attraction.

Weather data for each collection date was obtained from the Alabama Weather Information Services (AWIS) from a station that was approximately ten kilometers from the study site.
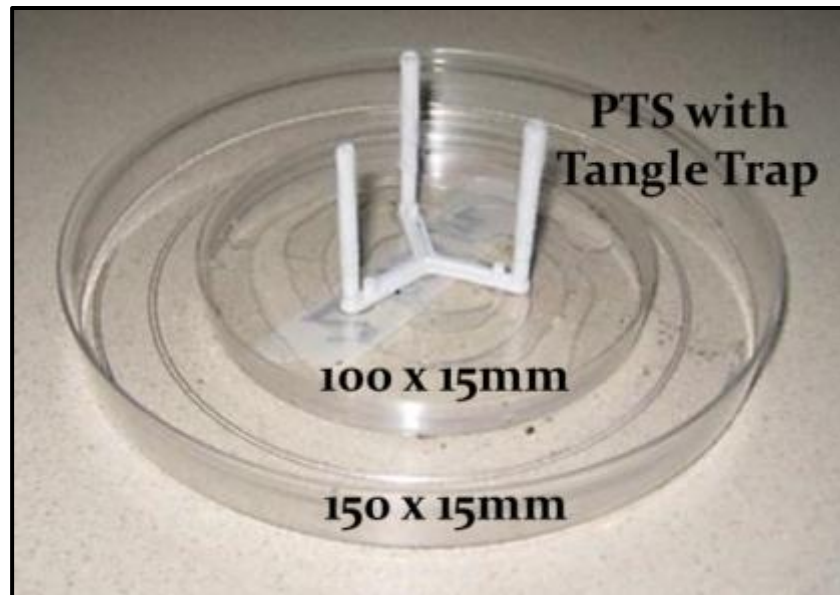
Fig. 7. Trap design that was used to monitor for *Pseudacteon curvatus* and *P. tricuspis* seasonal abundance patterns. Frozen fire ants placed into the interior Petri dish served as the phorid attractant.

Results

A total of 1,480 traps were analyzed that covered 148 collection periods. Over this period a total of 7,284 *P. curvatus* were collected and only 23 *P. tricuspis* were collected. *Pseudacteon curvatus* was found to be most abundant between 9 June and 22 August of 2012 (Fig. 8). Over this period an average of 21.8 *P. curvatus* were collected per sampling period. Ambient temperature averaged 26.6 °C over these collection periods. *Pseudacteon curvatus* was not collected between 12 December 2012 and 15 April 2013, when temperatures averaged 11.1 °C (raw data in Appendix 5).

Pseudacteon tricuspis was found on eight collection dates in 2012 (18, 20, 22, 25, 27, 29 June; 7 and 17 September), and only 23 *P. tricuspis* were collected over these dates. The highest period of seasonal abundance was between 18-29 June 2012. Over this period an average of 0.3 *P. tricuspis* were collected per sampling date. Ambient temperature averaged 26.3 °C during the period of highest abundance.
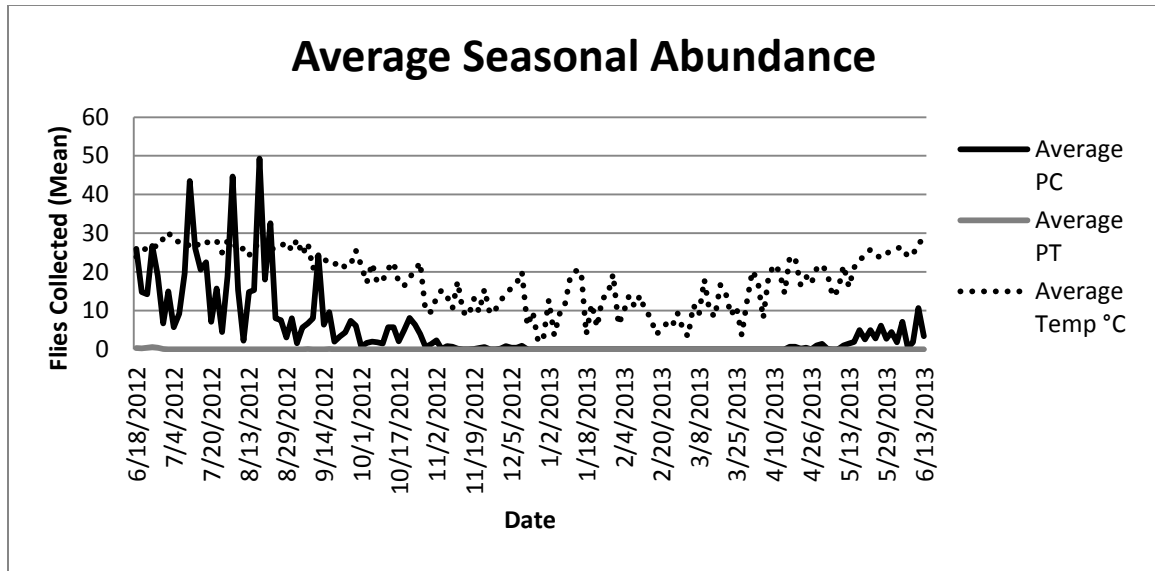
Fig. 8. Average seasonal abundance of *Pseudacteon curvatus* (PC) and *P. tricuspis* (PT) in relation to ambient temperature, in Alabama.

Discussion

Average seasonal abundance was observed for *P. tricuspis* while it was co-established with several other *Pseudacteon* spp. in South America. *Pseudacteon tricuspis* was found throughout the year, but flight activity ceased when ambient temperatures dropped below 20 °C (Calcaterra et al. 2008). However, at that location, the temperatures rarely dropped below this point. This supports the original study in South America that also found *P. tricuspis* throughout the year when co-established with other phorid species (Pesquero et al. 1996). *Pseudacteon tricuspis* was found to be active throughout the year when it was studied in Florida when it was independently established, and was only limited when ambient temperatures dropped below 20 °C (Morrison & Porter 2005). In Florida, *P. tricuspis* was found to have peaks in overall abundance in November, March, June and July, with the highest densities occurring in November (Morrison & Porter 2005).

When *P. tricuspis* was independently established in Alabama in 2002, it was found to be most abundant in June and July (Bertagnolli & Graham 2004). However, the data collected in the 2012-2013 study was more extensive from this and all other previous seasonal abundance studies of *P. tricuspis*. When *P. tricuspis* is co-established with *P. curvatus*, there is a shift in seasonal abundance and overall population density. *Pseudacteon tricuspis* was only found in June of 2012 in the year-long study. During this time it was found only in limited abundance. Temperatures regularly dropped below 20 °C in the fall and winter months during the 2012-2013 study, and flight activity ceased when temperatures reached these levels. However, this factor cannot be attributed to the reduction in *P. tricuspis* numbers throughout the rest of the study period when

temperatures averaged 21.7 °C. These data add support to the trends observed in the diurnal flight study, that *P. curvatus* appears to be more successful than *P. tricuspis* when the two species are co-established.

Pseudacteon curvatus was found to have sporadic patterns of abundance in South America when it was co-established with other phorid species. This was hypothesized to be due to competition with another phorid species, *P. cultellatus,* for the smaller fire ant workers (Calcaterra et al. 2008). *Pseudacteon curvatus* and *P. cultellatus* overlap slightly in host size preference, with *P. cultellatus* preferring the smallest fire ant workers.

In the United States, seasonal abundance patterns of *P. curvatus* were studied in Alabama in 2002. At that time and location, *P. curvatus* did not co-occur with any other introduced *Pseudacteon* species. *Pseudacteon curvatus* was found to be most abundant between July and August (Bertagnolli & Graham 2004; Bertagnolli & Graham 2005). This pattern was supported by the 2012-2013 study. This data suggests that the best time to study *P. curvatus* in the field would be between July and August in Alabama.

These data suggest very interesting interactions between *P. curvatus* and *P. tricuspis* once they are co-established in the United States, which were discussed in the previous study. *Pseudacteon curvatus* population densities are now nearly 320 times larger than densities of *P. tricuspis* where co-established in Alabama. This finding will be important when considering new phorid species for introduction. It may be pointless to introduce new species of phorid that occupy similar host size preferences, seasonal/diurnal patterns, and host semiochemicals attractants as *P. curvatus* if *P. curvatus* is already established and dominant in the location.

IV. Evaluation of trapping methods for *Pseudacteon curvatus*, *Pseudacteon litoralis* and *Pseudacteon tricuspis* (Diptera: Phoridae) in Alabama

Background and Significance

Pseudacteon litoralis was released into Alabama, Florida, Mississippi and Louisiana in the U.S., but was only successfully established in Alabama (Porter et al. 2011). This species was released in Wilcox County, Alabama in 2005 and was well established along with *P. curvatus* and *P. tricuspis*, at this release site in 2007. Today, *P. litoralis* has spread and is now found in Butler, Dallas, Lowndes and Wilcox counties in Alabama (Fig. 2).

Researchers believe that this species has continued to spread into other Alabama counties, but we have been unable to determine this due to a lack in travel funding. The area where *P. litoralis* is established is approximately a 2.5 h drive from our lab in Auburn, Alabama. In order to monitor the outward spread of *P. litoralis*, trapping methods were tested that would allow for more efficient data collection. Continued research into this species is important as we are observing interesting changes in the interactions between multiple *Pseudacteon* spp. when they become co-established. Alabama has a unique opportunity to contribute valuable research on *P. litoralis*. With a time and cost effective trapping method this data would be more easily obtained.

I hypothesized that the same trapping method that was successful at trapping *P. curvatus* and *P. tricuspis* would be successful at trapping *P. litoralis*. Both *P. curvatus* and *P. tricuspis* have only been observed around disturbed fire ant mounds and are attracted to very similar fire ant semiochemicals for oviposition (Chen et al. 2009;

Sharma et al. 2013). *Pseudacteon litoralis* has also only been observed around disturbed

fire ant mounds (Porter 1998), which leads to the belief that similar fire ant

semiochemicals are used as attractants for oviposition. Therefore, the same trapping

method should be successful for *P. curvatus, P. tricuspis* and *P. litoralis*.

Materials and Methods

The study was conducted at the farm of Walt and Peggy Prevatt (32°01'03.36 N,

87° 00'30.95 W) in Wilcox County, Alabama. This site is approximately ten kilometers

from the original *P. litoralis* release site. *Pseudacteon curvatus, P. litoralis* and *P.*

tricuspis have been co-established since 2007. This site allowed us to obtain and compare

trapping results for *P. curvatus, P. litoralis* and *P. tricuspis*.

A three plot system was created to evaluate the trapping methods. Each plot

measured 100 x 100 m, with a collection method placed in the center of each plot.

Phorids have been found to be attracted to host fire ants at distances of less than 50 m

(Morrison et al. 1999). Therefore, traps were placed 100 m apart so as not to get

competition between trapping methods (Sanford Porter, USDA-APHIS, Gainesville, FL,

personal communication). The plots were laid out in such a way as to have the traps

located in the shade and the wind movements minimalized. The centers of each plot

(where the traps were to be placed for monitoring) were marked and numbered with

flags. These locations were also marked using a hand held GPS unit, which served as a

back-up plan in the event that the flags were lost.

Three trap types were tested: (1) a trap with 2 g of freshly frozen fire ants as the

phorid attractant, (2) a trap with 2 g of only large sized (4-6 mm) freshly frozen fire ants

used as the phorid attractant, and (3) the tray trapping method, which was the same as was described in the diurnal oviposition study (Fig. 9). This tray trapping method is the standard method used to monitor for *P. litoralis* in the field. The trap with only large workers was selected because *P. litoralis* is known to attack the largest workers in the fire ant mound, and it was unknown if using only large workers would aid in collection of this species. The trap with all size fire ant workers as the phorid attractant was used because it is the standard for monitoring for *P. curvatus* and *P. tricuspis* (Puckett et al. 2007). Both the trap with all size workers and the trap with only large workers were designed in the same manner as described in the seasonal abundance objective above.

Fire ants were obtained from outside of the study area, but within eight kilometers of the site. Fire ants were excavated, placed into five gallon buckets, and separated from the soil in the same manner as described in the two previous objectives. Two fire ant colonies were obtained for each study period. After the fire ants were separated from the soil, one colony was placed into a tray, the additional moisture was allowed to dry, and the tray was placed in the freezer overnight, to kill the ants. The frozen ants were used for the two trap methods tested. When the other colony was separated from the soil, 40 g of ants were weighed, placed in a tray and kept alive for the study (in the same manner as in the diurnal oviposition study).

The PTS traps and the tray of ants were transported to the field one day after the ants were separated from the soil. The traps were placed in the field at noon, left out for 24 h, and collected the following day at noon. The tray of live ants was also placed out at noon, and flies were collected every 30 min until dark. This was then continued from sunrise the next day until noon. Data were collected and recorded for the tray method in

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the same manner as in the diurnal oviposition study. The traps were taken back to the lab where flies were identified to species, sexed and the data recorded.

The study was conducted between 31 Jun and 2 Oct 2013 for a total of 10 sample periods (replicates). Tray locations were randomly assigned prior to each trapping date.

Data were analyzed using generalized mixed model analysis using LS MEANS with $P = 0.05$ (SAS PROC GLIMMIX, SAS Version 9.2, Copyright® 2014 SAS Institute Inc., Cary, NC). Trap type effectiveness for each species was analyzed with $P = 0.05$.
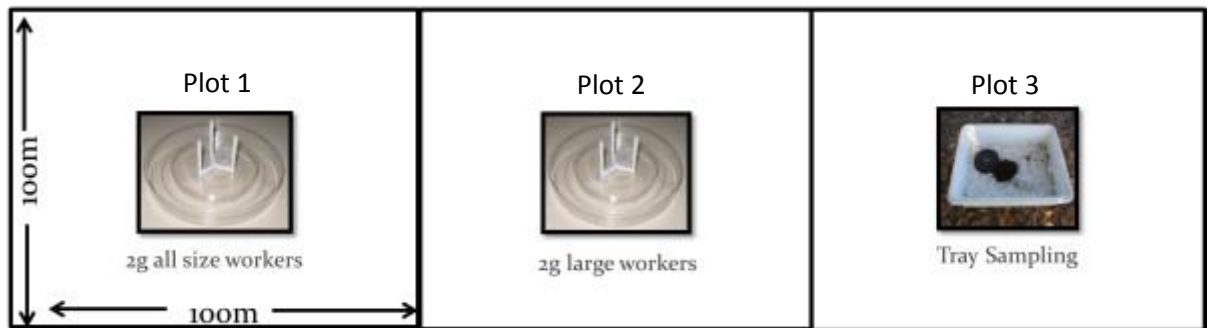
Fig. 9. Experimental design used for evaluating the trapping methods of *Pseudacteon curvatus*, *Pseudacteon litoralis,* and *Pseudacteon tricuspis*.

Results

Pseudacteon curvatus collections differed (df = 72, $P < 0.05$) due to trap type. A mean of 23.2 *P. curvatus* were collected from the trap with all size workers, this was not significantly different from the trap with only large size workers, which collected a mean of 26.6 *P. curvatus* (Fig. 10). In comparison, a mean of 15.5 *P. curvatus* were collected using the tray sampling method, which was significantly less than the trap with all size workers and the trap with only large workers. Overall, more *P. curvatus* were collected regardless of trapping method used than *P. litoralis* and *P. tricuspis* (df = 72, $P < 0.05$).

Pseudacteon tricuspis collections differed (df = 72, $P < 0.05$) due to trap type (Fig. 10). A mean of 3.0 *P. tricuspis* were collected with the trap with all size workers, a mean of 3.5 *P. tricuspis* were collected with the large size worker trap, these trapping methods were not significantly different. Significantly fewer *P. tricuspis*, a mean of 1.1, were collected using the tray method.

Pseudacteon litoralis collections differed (df = 72, $P < 0.05$) due to trap type (Fig. 10). A mean of 1.7 *P. litoralis* were collected using the trap with all size workers, a mean of 2.2 *P. litoralis* were collected with the trap with only large workers, and a mean of 3.8 *P. litoralis* were collected using the tray method. Significantly more *P. litoralis* were collected using the tray method than were collected using the trap with all size workers. However, there were no significant differences between the trap with all size workers and the trap with only large size workers. Also, there was no significant difference between the tray method and the trap with only large size workers.

Raw data collected for this study can be found in appendix six at the end of this document.
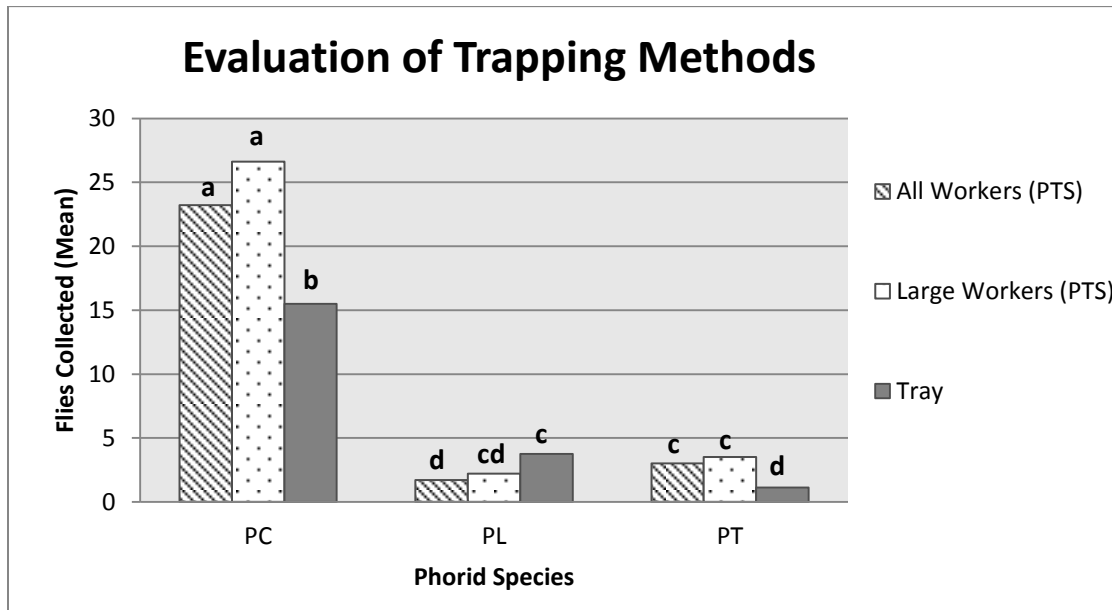
Fig. 10. Evaluation of the trapping methods tested for *Pseudacteon curvatus*, *Pseudacteon litoralis,* and *Pseudacteon tricuspis,* Wilcox Co., 2013. Different letters indicate means that are significantly different ($P < 0.05$).

Discussion

All trapping methods were successful at monitoring for the presence of *P. curvatus*. The data suggests that both the trap with all size workers and the trap with large size workers would be highly successful at trapping *P. curvatus* in the field. This confirms what was already established by Puckett in 2007.

Just as in the previous two objectives, *P. tricuspis* was only found in limited numbers. Fewer *P. tricuspis* were collected using the tray trapping method, similar to the results found in Texas (Puckett et al. 2007). There were no differences among the PTS trapping methods tested, which suggests that either would be successful in the field. However, with such limited numbers it may be difficult to pick up on the presence of *P. tricuspis* in the field. In future studies, if the presence of *P. tricuspis* were the objective, it would be necessary to increase the amount of traps used. This data provides even more evidence to the fact that *P. tricuspis* population numbers are declining when it is co-established with other phorid species, as was noted in the previous studies.

Pseudacteon litoralis was found in low numbers with all trap types tested, however, it was found in slightly higher numbers with the tray collection technique. One problem with this data analysis was that the only way to compare between trap types systematically was to compare the numbers on the PTS traps to the average collected over the 24 h period with the tray trapping method. However, with the tray trapping method the data was skewed due to the zeroes recorded in the middle of the day when *P. litoralis* is known to be inactive. If this study could be conducted again, the PTS traps as well as the tray trapping method should have only been tested during periods of known *P.*

litoralis diurnal activity (early morning and late afternoon). This would have provided a better data set for comparison.

When the raw data is examined, the tray trapping method appears to be more successful than either PTS trap type for *P. litoralis*, which leads us to believe that there may be another factor used by *P. litoralis* in host location that differs from both *P. curvatus* and *P. tricuspis*. In addition, since the PTS trapping methods were only successful at collecting a limited number of *P. litoralis*, they would not be viable options for monitoring the outward spread of this species. If *P. litoralis* are found in limited numbers on the outward edge of their range, the most successful monitoring method needs to be employed in order to determine the presence of small populations. More studies need to be conducted into the exact host location methods of *P. litoralis,* as these will be helpful in developing a trap that will successfully monitor for this species. Also, a follow-up study should be conducted which examines the PTS trap types to the tray trapping method during periods of known *P. litoralis* activity. This would allow a better understanding of the differences between trap types.

V. Parasitism rates of *Pseudacteon curvatus* and *Pseudacteon tricuspis* (Diptera: Phoridae), parasitoids of *Solenopsis* spp. (Hymenoptera: Formicidae) fire ants in Alabama

Background and Significance

The parasitism rates by *Pseudacteon* spp. co-existing in South America was found to be quite low, although no actual parasitism rates were given (Jouvenaz at al. 1981). In Florida, when only *P. tricuspis* was established, parasitism rates were also found to be low, always < 1% (Morrison & Porter 2005). A study conducted in Texas observed parasitism rates for another host/parasitoid assemblage, native *Pseudacteon* spp. flies attacking the native fire ant species (*S. geminata*), found parasitism rates of less than 3% (Morrison et al. 1997).

Parasitism rates have never been observed for *P. curvatus* and *P. tricuspis* where they are co-established in the United States. In order to understand the effects of these parasitoids, continued research needs to be conducted to understand their impacts on fire ant populations. A study was conducted between May and Oct 2013 where these species are co-established in Alabama.

I hypothesized that there would be an increase in parasitism rates from those observed in Florida when *P. tricuspis* was independently established. With multiple species established that parasitize a greater size range of fire ant workers, percentage parasitism would be expected to increase.

Materials and Methods

Three sites were selected for this study; the first was the original *P. tricuspis* release site in Macon County, Alabama (Notasulga) at the farm of Tony and Diane Silva (32°34'26.55"N, 85°39'55.03"W). The second site was located in Lee County, Alabama (Loachapoka) at the farm of Phil and Daria Story (32°34'36.33"N, 85°35'42.58"W), and the final site was in Lee County, Alabama (Auburn), behind the Auburn University Beef Unit (32°35'20.74"N, 85°29'40.93"W). Both *P. curvatus* and *P. tricuspis* were established at each location since 2007. The study was conducted in May, Jun, Aug and Oct of 2013.

Ten mounds from each site were excavated on each sampling date. Mounds were selected at random, but were at least 0.3 x 0.3 m in size. Fire ants were excavated, transported, and separated from the soil as in the previous objectives. Once separated from the soil, a random subsample of 2 g of fire ant workers (Calcaterra et al. 2008) were weighed and placed into individual 33 x 18 x 10 cm Sterilite® plastic storage containers that were lined with Fluon® (Asahi Glass Ltd, Chadds Ford, PA) to prevent ant escape. This 2 g subsample was selected because it was the same amount of ants used to determine parasitism rates in both South America and Florida; therefore, it was necessary to use the same amount for comparative purposes. The 2 g sample was found to contain approximately 18,750 fire ant workers (Vicky E. Bertagnolli, Clemson University Extension, Clemson, SC, personal communication). Each container was labeled by date and location. The lids of the plastic containers were modified and a hole was cut and covered with NoSeeum Insect Netting® (Balson Hercules, Providence, RI) (Fig. 11). This allowed the boxes to get air flow when they were inside the growth chamber. Even air

flow was needed to prevent molding of the ant colony. Containers were then placed inside growth chambers and were kept at 25 °C-27 °C with a 12:12 h L:D photoperiod (Morrison and Porter 2005).

The ants were provided with a 15 x 95 mm plastic tube filled with water and another plastic tube filled with 20% sugar water. Both were stuffed with a cotton ball at the end, which allowed access to the contents of the tube by the ants without spilling liquids into the container. They were also provided a 5 cm diameter by 2.5 cm dental plaster block, which was routinely saturated by holding it underwater. The plaster block was then dried with a paper towel to eliminate excess moisture. The saturated plaster blocks maintained the humidity around 100% inside the growth chamber throughout the study period (Morrison & Porter 2004). Each tray was inspected every two days for 50 d for the appearance of pupae in the heads of dead ants (Morrison & Porter 2004). Phorid pupae that were found were transferred to new plastic containers until adult flies emerged. The plastic containers were modified in the same manner as those used for the fire ant samples. Fly pupae were placed on 5 x 2.5 cm plaster blocks that were saturated as described above, which prevented desiccation of the pupae. Flies that emerged were identified to species and sexed. Parasitism rates were determined by dividing the total number of flies per tray by the total number of fire ant workers per tray, and this number was multiplied by 100.

Parasitism, temperature, rainfall and fire ant density data were analyzed using regression techniques within a generalized linear model framework with a binary distribution function with $P = 0.05$ (SAS PROC GLIMMIX, SAS Version 9.4,

Copyright® 2014 SAS Institute Inc., Cary, NC). Location day means were analyzed with

$P = 0.05$.

Fig. 11. Modified boxes containing ants inside the growth chamber during the parasitism rate study.

Results

A total of 133 flies were collected from approximately 225,000 fire ant workers (raw data in Appendix 7). This yielded a mean parasitism rate over all sampling dates of 0.059% (Fig. 12). All fly pupae that were identified in fire ant head capsules emerged as adults, yielding a 100% survival rate of pupae. All flies that emerged were *P. curvatus* females. No *P. curvatus* males and no *P. tricuspis* were found during the parasitism study.

There were significant differences observed between dates and sites sampled ($P < 0.05$). The highest parasitism rates were observed in October, where rates averaged 0.084%. Rates were similar between all sites for each month of data collection; however, rates at the Story Farm in Loachapoka, Lee County, Alabama, were significantly less than the other two sites in May and October. Parasitism rates were significantly higher in August and in October 2013 at the Silva Farm in Notasulga, Macon County, Alabama, where means of 0.091% were observed for each sampling period. In June 2013, parasitism rates were significantly higher at the Auburn University Beef Unit, Auburn, Alabama (0.08%) than the other two sites. There were no significant differences between the Auburn University Beef Unit, Auburn, Alabama, and the Silva Farm, Notasulga, Alabama, in May 2013 ($P \geq 0.05$). These differences in parasitism rate were not correlated with any environmental factors, such as temperature, rainfall, or fire ant densities ($P \geq 0.05$).

Phorid pupae were not observed in the lab for the first 10 d after collection of the ants. This held true for all of the locations and dates of sampling. Phorid pupae were

found consistently from day 11 through day 45 after collection for all sites. No pupae were found after day 45.
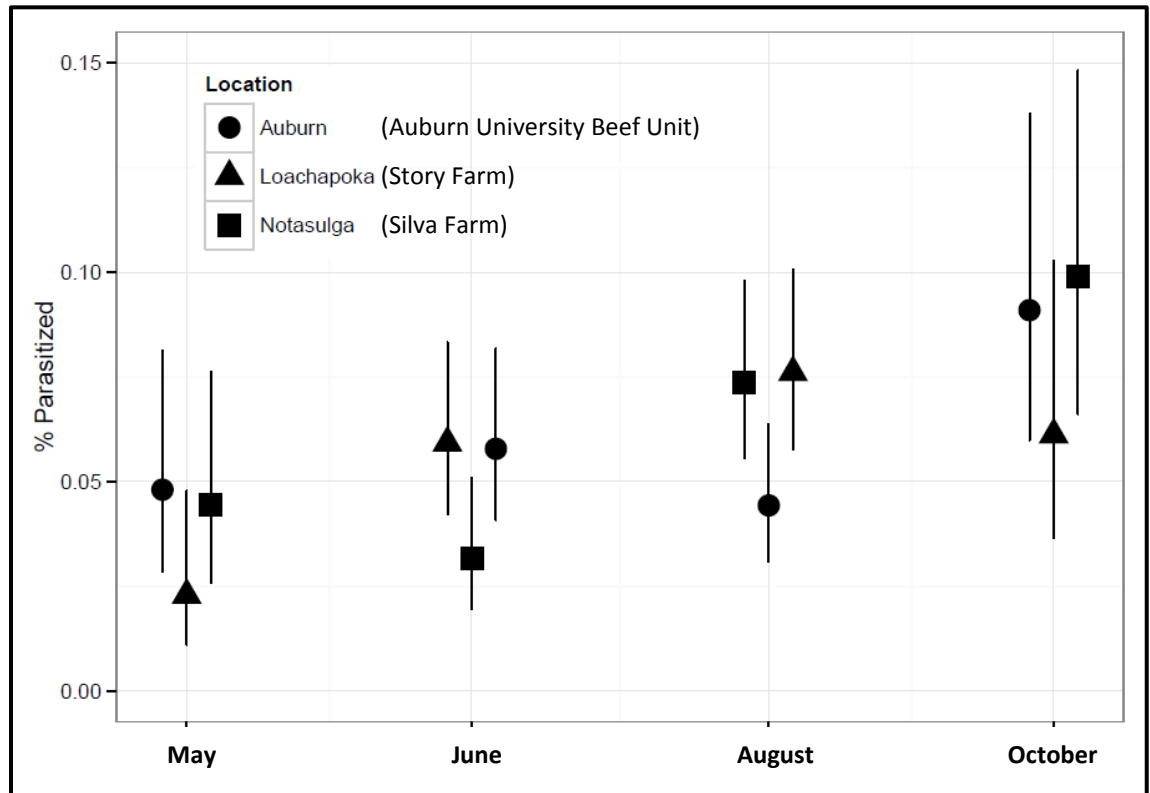
Fig. 12. Percent parasitism of imported fire ants by *Pseudacteon* spp. flies by date and field site. The highest overall observed rates occurred in Aug 2013. Rates were significantly less at the Story farm in May/October. Rates were significantly higher at the Silva farm in August/October. Rates were significantly higher at the Auburn University beef unit in June. Bars indicate range between upper and lower limit of parasitism rates observed for each site/date.

Discussion

All parasitism rates in this study were < 1%. The overall rates observed in Florida for *P. tricuspis* in 2001 were 0.058% (Morrison & Porter 2005), and similarly the overall rates observed in this study were 0.059%. This disproved my hypothesis that an increased rate of parasitism would occur with multiple species established. However, only *P. curvatus* were collected, which adds evidence of the limited presence of *P. tricuspis* in the environment when these species are co-established. With only small populations of *P. tricuspis* around these study areas it makes sense that we would see parasitism rates that are similar to those collected in Florida, when only one species was established. A larger sample size might have been needed to detect *P. tricuspis*.

Even with multiple species co-existing in South America, the reports of parasitism are quite low (Jouvenaz at al. 1981). These studies continue to provide support for the indirect effects that *Pseudacteon* spp. have on *Solenopsis* spp. fire ants. In South America as well as in the United States, behavioral changes were observed when fire ants were in the presence of *Pseudacteon* spp. flies (Morrison 1999). Fire ant workers were documented to decrease foraging activities and decrease mound-rebuilding efforts when in the presence of *Pseudacteon* spp. parasitoids (Feener & Brown 1992; Orr et al. 1995; Porter et al. 1995b; Morrison 1999). These indirect effects cause an overall decrease in the health of the colony and can cause colony collapse and death (Feener & Brown 1992).

These effects have important implications for the fire ant biological control program. If new phorid species are introduced, the focus should be on *Pseudacteon* spp. that utilize different diurnal, seasonal, host size, and attraction semiochemical preferences

than those already established. This could provide increased pressure on fire ants and could eventually create decreases in United States fire ant population densities.

In the 2013 parasitism study, no pupae were found prior to day 10 after collection of the ant colonies. This was also found in Florida, where no pupae were found between day six and day 10 after collection (Morrison & Porter 2005). This provides more evidence of the behavioral changes (zombie ants) that occur in the fire ant host just prior to the decapitation of the ant. Parasitized ants were observed leaving the colony during this period, thus, completing their life cycle outside of the fire ant colony (Henne & Johnson 2007). This would explain the lack of pupae found before day 10 in our study. Also, no phorid pupae were found after day 45, which supports the findings that pupariation in *Pseudacteon* spp. begins around day 11 after oviposition and ends around day 42 (Morrison & Porter 2005).

In addition, only female *P. curvatus* were collected. This could point to a disproportion in the size of the fire ant workers in the colonies sampled. The sex of *Pseudacteon* spp. flies is determined by the size of the host ant parasitized (Morrison et al. 1999). Female flies typically emerge from fire ant workers that are on the larger end of the host size preference scale, whereas, male flies emerge from fire ant workers on the smaller end of the scale. Therefore, the data may have been skewed due to a higher abundance of larger fire ant workers (≥ 0.71 mm in head width) in the colonies sampled. However, head width was not measured in this study, so the exact reason is unknown.

The findings in this parasitism study support the other previous studies on *Pseudacteon* spp., with all rates remaining low, $< 1\%$ (Morrison & Porter 2005). The most important finding was perhaps the lack of collection of *P. tricuspis* in this study.

Again, this is most likely attributed to the low population density of P. tricuspis, which was even more skewed due to small sample size.

An important note is that natural parasitism rates are thought to be higher than those observed in this study as well as those observed in all previous studies (Morrison & Porter 2005). This was attributed to the low sample size (2 g) of fire ant workers used in these studies. However, it is extremely difficult to increase the sample size for this study due to the amount of laboratory space that would be required.

These findings are extremely important for the future and overall success of the fire ant biological control program. In order for the program to be successful, any new species of phorid fly that is introduced where another species is currently established should occupy a different seasonal, diurnal, host size, and/or semiochemicals attraction preference. This will better equip the new species for success when another species is already established.

VI. Conclusions & future research implications

Since their overlap in geographic distribution in Alabama, *P. tricuspis* population numbers have drastically declined compared to *P. curvatus* numbers. This trend was also observed in both Florida and Texas, although no formal research into this was conducted. This decline could be due to several factors, including: interspecific competition due to overlap in diurnal cycle, a higher abundance of hosts for *P. curvatus*, differences in climatic variables (primarily days below 20 °C) between the U.S. and South America, faster developmental time for *P. curvatus*, and differences in the amount of behavioral change in host fire ants when in the presence of *P. curvatus* allowing for longer ovipositional cycles. These factors could be acting alone, or in any combination to aid in the successfulness of *P. curvatus*. There could also be any number of other factors that interact with those listed above, or that are currently unknown that are attributing to the lack of success of *P. tricuspis* in the U.S.

Additional research needs to be conducted into the exact interactions that are attributing to the decline in *P. tricuspis* numbers. Future studies into differences in climate are of particular interest especially in states with different temperature regimes than those observed in South America. Also, in states where multiple phorid species are not co-established, it will be important to monitor yearly as the different species spread in the hopes of observing causes/sources of decline in population numbers when multiple species become established in the same location.

This future research will be pivotal as we continue to release new phorid species into U.S. fire ant populations. The interactions among species could affect the establishment of new phorid species and could change existing phorid release protocols.

Primarily, it will be important to release species that exploit different niches than the

species that are already established in a location. With further research, we will be better

equipped to continue the biological control program for imported fire ants in the U.S.

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Appendices

Appendix 1. Diurnal patterns of ovipositional activity when *P. curvatus* (PC) and *P. tricuspis* (PT) are co-established, Lee Co., 2010 (raw data)

Time (Hrs after sunrise)	Phorids Collected (Mean)	
	PC	PT
0.5	0.0	0.0
1.0	0.0	0.0
1.5	0.1	0.0
2.0	2.3	0.0
2.5	6.6	0.1
3.0	6.6	0.1
3.5	9.0	0.0
4.0	9.3	0.0
4.5	8.3	0.0
5.0	8.3	0.3
5.5	7.9	0.0
6.0	3.7	0.0
6.5	3.2	0.0
7.0	4.0	0.3
7.5	4.3	0.2
8.0	4.3	0.2
8.5	4.5	0.2
9.0	4.5	0.2
9.5	5.7	0.2
10.0	6.8	0.0
10.5	7.3	0.5
11.0	6.8	0.3
11.5	7.7	0.2
12.0	7.8	0.2
12.5	6.7	0.0
13.0	7.2	0.0
13.5	2.0	0.0
14.0	1.0	0.0

Appendix 2. Diurnal patterns of ovipositional activity when *P. curvatus* (PC) and *P. tricuspis* (PT) are co-established, Lee Co., 2012-2013 (raw data)

Time (Hrs after sunrise)	Phorids Collected (Mean)	
	PC	PT
0.5	0.0	0.0
1.0	0.1	0.0
1.5	0.3	0.0
2.0	1.0	0.0
2.5	1.8	0.0
3.0	3.1	0.0
3.5	4.4	0.0
4.0	9.1	0.0
4.5	10.6	0.0
5.0	5.6	0.0
5.5	5.7	0.0
6.0	4.5	0.0
6.5	4.6	0.0
7.0	4.1	0.0
7.5	3.5	0.0
8.0	2.9	0.0
8.5	2.5	0.0
9.0	2.5	0.0
9.5	2.7	0.0
10.0	2.8	0.0
10.5	3.6	0.0
11.0	5.8	0.0
11.5	6.4	0.0
12.0	6.8	0.0
12.5	5.8	0.0
13.0	4.2	0.0
13.5	1.8	0.0
14.0	0.7	0.0

Appendix 3. Diurnal patterns of ovipositional activity when *P. curvatus* (PC), *P. litoralis* (PL) and *P. tricuspis* (PT) are co-established, Wilcox Co., 2010 (raw data)

Time (Hrs After Sunrise)	Phorids Collected (Mean)		
	PC	PT	PL
0.5	0.0	1.0	0.5
1.0	0.0	0.0	0.7
1.5	0.0	3.0	22.8
2.0	0.2	3.6	19.6
2.5	0.6	3.6	22.0
3.0	0.4	3.2	10.6
3.5	1.2	1.2	6.4
4.0	2.0	2.0	3.0
4.5	2.4	2.2	0.6
5.0	1.4	1.4	0.2
5.5	3.4	1.6	0.0
6.0	3.8	3.0	0.0
6.5	4.2	1.6	0.0
7.0	5.4	1.4	0.0
7.5	5.8	1.6	0.0
8.0	5.2	1.4	0.0
8.5	6.3	0.5	0.0
9.0	9.7	2.2	0.0
9.5	11.7	3.5	0.0
10.0	16.2	2.4	0.0
10.5	15.0	3.4	0.0
11.0	12.2	2.4	0.0
11.5	13.2	2.8	0.0
12.0	9.5	2.5	0.0
12.5	8.3	2.2	0.5
13.0	3.0	1.2	2.2
13.5	1.5	0.0	0.3
14.0	0.0	0.0	0.3

Appendix 4. Diurnal patterns of ovipositional activity when *P. curvatus* (PC), *P. litoralis* (PL) and *P. tricuspis* (PT) are co-established, Wilcox Co., 2013 (raw data)

Time (Hrs After Sunrise)	Phorids Collected (Mean)		
	PC	PT	PL
0.5	0.0	0.0	0.4
1.0	0.0	0.0	5.0
1.5	0.2	0.0	8.6
2.0	0.5	0.1	16.8
2.5	0.6	0.4	17.0
3.0	1.6	0.4	16.3
3.5	3.1	0.3	10.3
4.0	6.4	0.7	4.3
4.5	11.4	1.5	0.9
5.0	20.0	1.0	0.2
5.5	23.1	1.1	0.1
6.0	27.1	2.3	0.0
6.5	31.2	2.1	0.0
7.0	33.6	2.7	0.0
7.5	37.6	2.3	0.0
8.0	36.7	2.0	0.0
8.5	34.2	2.1	0.0
9.0	28.5	2.0	0.0
9.5	25.7	1.4	0.1
10.0	18.7	1.7	0.4
10.5	15.0	1.3	1.1
11.0	9.9	0.9	1.9
11.5	4.5	0.0	2.4
12.0	4.0	0.0	4.3
12.5	3.8	0.0	6.3
13.0	2.0	0.0	3.7
13.5	3.0	0.0	1.0
14.0	0.0	0.0	0.0

Appendix 5. Average seasonal abundance of *P. curvatus* (PC) and *P. tricuspis* (PT) in relation to ambient temperature, in Alabama (raw data)

Date	Phorids Collected (Mean)		High Temp. °C	Low Temp. °C	Average Temp °C
	PC	PT			
6/18/2012	26.0	0.3	30.0	17.8	23.9
6/20/2012	14.8	0.2	31.1	20.0	25.6
6/22/2012	14.2	0.4	32.2	20.0	26.1
6/25/2012	26.7	0.6	32.2	21.7	26.9
6/27/2012	19.0	0.4	33.9	18.9	26.4
6/29/2012	6.7	0.1	35.0	22.2	28.6
7/2/2012	15.0	0.0	38.9	21.7	30.3
7/4/2012	5.7	0.0	36.1	20.0	28.1
7/6/2012	9.2	0.0	35.0	20.6	27.8
7/9/2012	19.6	0.0	32.8	22.8	27.8
7/11/2012	43.5	0.0	32.2	21.1	26.7
7/13/2012	26.1	0.0	31.1	21.1	26.1
7/16/2012	20.6	0.0	33.3	22.8	28.1
7/18/2012	22.5	0.0	33.9	21.7	27.8
7/20/2012	7.1	0.0	31.7	22.8	27.2
7/23/2012	15.7	0.0	33.3	22.8	28.1
8/1/2012	4.5	0.0	28.3	21.7	25.0
8/3/2012	18.9	0.0	35.0	21.1	28.1
8/6/2012	44.7	0.0	32.8	23.3	28.1
8/8/2012	15.3	0.0	27.8	22.8	25.3
8/10/2012	2.2	0.0	31.1	21.1	26.1
8/13/2012	14.8	0.0	30.6	17.8	24.2
8/15/2012	15.3	0.0	32.2	21.1	26.7
8/17/2012	49.3	0.0	32.8	21.1	26.9
8/20/2012	18.0	0.0	27.8	21.7	24.7
8/22/2012	32.6	0.0	31.1	21.1	26.1
8/24/2012	8.0	0.0	31.7	20.0	25.8
8/27/2012	7.5	0.0	32.2	21.1	26.7
8/29/2012	3.1	0.0	32.2	23.3	27.8
8/31/2012	8.0	0.0	28.3	22.8	25.6
9/3/2012	1.6	0.0	32.8	23.9	28.3
9/5/2012	5.6	0.0	26.7	22.2	24.4
9/7/2012	6.7	0.1	32.8	22.2	27.5
9/10/2012	8.0	0.0	27.2	14.4	20.8
9/12/2012	24.3	0.0	29.4	19.4	24.4
9/14/2012	6.4	0.0	28.9	18.3	23.6
9/17/2012	9.6	0.1	27.8	16.1	21.9

	Phorids Collected (Mean)			Low Temp.	Average
Date	PC	PT	High Temp. °C	°C	Temp °C
9/19/2012	2.0	0.0	27.2	16.7	21.9
9/21/2012	3.3	0.0	28.9	16.7	22.8
9/24/2012	4.4	0.0	28.9	13.3	21.1
9/26/2012	7.4	0.0	28.3	15.6	21.9
9/28/2012	6.1	0.0	31.7	19.4	25.6
10/1/2012	0.7	0.0	23.3	20.0	21.7
10/3/2012	1.7	0.0	21.7	12.8	17.2
10/5/2012	2.0	0.0	28.3	16.1	22.2
10/8/2012	1.8	0.0	22.8	11.7	17.2
10/10/2012	1.5	0.0	23.3	12.2	17.8
10/12/2012	5.7	0.0	27.2	14.4	20.8
10/15/2012	5.7	0.0	27.2	17.8	22.5
10/17/2012	2.1	0.0	23.9	10.6	17.2
10/19/2012	5.0	0.0	24.4	8.3	16.4
10/22/2012	8.1	0.0	26.1	11.1	18.6
10/24/2012	6.4	0.0	27.2	13.3	20.3
10/26/2012	4.0	0.0	28.3	16.7	22.5
10/29/2012	0.6	0.0	15.0	4.4	9.7
10/31/2012	1.3	0.0	15.6	3.9	9.7
11/2/2012	2.3	0.0	20.6	6.1	13.3
11/5/2012	0.1	0.0	23.9	6.7	15.3
11/7/2012	0.8	0.0	19.4	7.8	13.6
11/9/2012	0.7	0.0	17.8	3.3	10.6
11/12/2012	0.2	0.0	23.9	11.1	17.5
11/14/2012	0.0	0.0	16.1	3.3	9.7
11/16/2012	0.0	0.0	11.7	5.6	8.6
11/19/2012	0.1	0.0	18.9	7.2	13.1
11/21/2012	0.3	0.0	16.1	3.3	9.7
11/23/2012	0.6	0.0	23.3	7.8	15.6
11/26/2012	0.0	0.0	16.7	1.7	9.2
11/28/2012	0.0	0.0	15.0	5.6	10.3
11/30/2012	0.2	0.0	20.0	5.0	12.5
12/3/2012	0.8	0.0	23.3	5.6	14.4
12/5/2012	0.4	0.0	23.3	8.3	15.8
12/7/2012	0.4	0.0	22.8	11.7	17.2
12/10/2012	0.9	0.0	23.9	16.1	20.0
12/12/2012	0.0	0.0	8.9	3.3	6.1
12/14/2012	0.0	0.0	16.1	3.3	9.7
12/28/2012	0.0	0.0	6.1	-2.2	1.9
12/31/2012	0.0	0.0	10.0	-4.4	2.8

	Phorids Collected (Mean)			Low Temp. °C	Average Temp °C
Date	PC	PT	High Temp. °C		
1/2/2013	0.0	0.0	18.3	7.2	12.8
1/4/2013	0.0	0.0	8.3	-1.1	3.6
1/7/2013	0.0	0.0	13.3	5.0	9.2
1/9/2013	0.0	0.0	16.7	6.1	11.4
1/11/2013	0.0	0.0	20.0	17.2	18.6
1/14/2013	0.0	0.0	23.3	17.2	20.3
1/16/2013	0.0	0.0	24.4	16.1	20.3
1/18/2013	0.0	0.0	6.7	1.1	3.9
1/21/2013	0.0	0.0	18.9	4.4	11.7
1/23/2013	0.0	0.0	11.7	0.0	5.8
1/25/2013	0.0	0.0	18.9	7.2	13.1
1/28/2013	0.0	0.0	19.4	8.9	14.2
1/30/2013	0.0	0.0	24.4	14.4	19.4
2/1/2013	0.0	0.0	11.1	1.7	6.4
2/4/2013	0.0	0.0	16.7	2.8	9.7
2/6/2013	0.0	0.0	20.6	7.2	13.9
2/8/2013	0.0	0.0	13.3	8.9	11.1
2/11/2013	0.0	0.0	18.9	8.9	13.9
2/13/2013	0.0	0.0	12.2	7.8	10.0
2/15/2013	0.0	0.0	13.9	2.8	8.3
2/18/2013	0.0	0.0	10.0	-2.2	3.9
2/20/2013	0.0	0.0	12.2	-2.2	5.0
2/22/2013	0.0	0.0	12.8	0.6	6.7
2/25/2013	0.0	0.0	10.0	1.7	5.8
2/27/2013	0.0	0.0	13.9	5.0	9.4
3/1/2013	0.0	0.0	11.1	-0.6	5.3
3/4/2013	0.0	0.0	7.8	-1.1	3.3
3/6/2013	0.0	0.0	21.7	1.1	11.4
3/8/2013	0.0	0.0	16.7	1.1	8.9
3/11/2013	0.0	0.0	23.9	12.2	18.1
3/13/2013	0.0	0.0	16.7	3.3	10.0
3/15/2013	0.0	0.0	15.6	1.1	8.3
3/18/2013	0.0	0.0	20.6	12.8	16.7
3/20/2013	0.0	0.0	21.7	7.2	14.4
3/22/2013	0.0	0.0	13.3	4.4	8.9
3/25/2013	0.0	0.0	20.0	2.2	11.1
3/27/2013	0.0	0.0	8.3	-1.1	3.6
3/29/2013	0.0	0.0	19.4	5.0	12.2

	Phorids Collected (Mean)				
Date	PC	PT	High Temp. °C	Low Temp. °C	Average Temp °C
4/1/2013	0.0	0.0	25.0	15.6	20.3
4/3/2013	0.0	0.0	24.4	11.1	17.8
4/5/2013	0.0	0.0	10.0	6.1	8.1
4/8/2013	0.0	0.0	25.0	11.7	18.3
4/10/2013	0.0	0.0	29.4	14.4	21.9
4/12/2013	0.0	0.0	27.2	13.3	20.3
4/15/2013	0.0	0.0	16.7	12.2	14.4
4/17/2013	0.7	0.0	28.9	18.9	23.9
4/19/2013	0.7	0.0	27.8	19.4	23.6
4/22/2013	0.2	0.0	22.2	11.1	16.7
4/24/2013	0.4	0.0	25.0	12.8	18.9
4/26/2013	0.0	0.0	23.9	10.0	16.9
4/29/2013	1.0	0.0	25.0	16.7	20.8
5/1/2013	1.4	0.0	27.2	16.7	21.9
5/3/2013	0.1	0.0	21.7	17.2	19.4
5/6/2013	0.0	0.0	20.0	7.2	13.6
5/8/2013	0.0	0.0	20.6	10.0	15.3
5/10/2013	1.0	0.0	28.3	15.0	21.7
5/13/2013	1.5	0.0	24.4	7.8	16.1
5/15/2013	1.9	0.0	27.2	15.0	21.1
5/17/2013	5.0	0.0	28.3	18.3	23.3
5/20/2013	2.6	0.0	28.9	18.9	23.9
5/22/2013	5.0	0.0	31.7	20.0	25.8
5/24/2013	2.8	0.0	30.6	18.3	24.4
5/27/2013	6.1	0.0	30.6	16.7	23.6
5/29/2013	2.7	0.0	30.6	18.9	24.7
5/31/2013	4.5	0.0	30.6	20.6	25.6
6/3/2013	1.8	0.0	31.7	20.6	26.1
6/5/2013	7.1	0.0	32.8	20.0	26.4
6/7/2013	0.4	0.0	27.2	20.6	23.9
6/10/2013	1.8	0.0	27.8	21.1	24.4
6/12/2013	10.7	0.0	32.2	22.8	27.5
6/13/2013	3.4	0.0	33.3	23.3	28.3

Appendix 6. Evaluation of trapping methods for *P. curvatus* (PC), *P. litoralis* (PL), and *P. tricuspis* (PT) in Alabama (raw data)

Date	Trap Type	Phorid Type	Count
7/31/13	All Size Workers	PC	15
7/31/13	Large Workers	PC	25
7/31/13	Tray	PC	13.4
7/31/13	All Size Workers	PL	1
7/31/13	Large Workers	PL	4
7/31/13	Tray	PL	0.3
7/31/13	All Size Workers	PT	2
7/31/13	Large Workers	PT	5
7/31/13	Tray	PT	2.4
8/7/13	All Size Workers	PC	15
8/7/13	Large Workers	PC	14
8/7/13	Tray	PC	17.8
8/7/13	All Size Workers	PL	2
8/7/13	Large Workers	PL	3
8/7/13	Tray	PL	2.2
8/7/13	All Size Workers	PT	8
8/7/13	Large Workers	PT	6
8/7/13	Tray	PT	2
8/14/13	All Size Workers	PC	22
8/14/13	Large Workers	PC	18
8/14/13	Tray	PC	13.6
8/14/13	All Size Workers	PL	4
8/14/13	Large Workers	PL	6
8/14/13	Tray	PL	7.6
8/14/13	All Size Workers	PT	7
8/14/13	Large Workers	PT	9
8/14/13	Tray	PT	1.2
8/20/13	All Size Workers	PC	18
8/20/13	Large Workers	PC	22
8/20/13	Tray	PC	11.5
8/20/13	All Size Workers	PL	2
8/20/13	Large Workers	PL	3
8/20/13	Tray	PL	2.8
8/20/13	All Size Workers	PT	3
8/20/13	Large Workers	PT	6
8/20/13	Tray	PT	1
8/27/13	All Size Workers	PC	32
8/27/13	Large Workers	PC	28
8/27/13	Tray	PC	14.1
8/27/13	All Size Workers	PL	3
8/27/13	Large Workers	PL	1

Date	Trap Type	Phorid Type	Count
8/27/13	Tray	PL	1.3
8/27/13	All Size Workers	PT	5
8/27/13	Large Workers	PT	3
8/27/13	Tray	PT	0.8
9/3/13	All Size Workers	PC	23
9/3/13	Large Workers	PC	32
9/3/13	Tray	PC	18.3
9/3/13	All Size Workers	PL	1
9/3/13	Large Workers	PL	2
9/3/13	Tray	PL	1.2
9/3/13	All Size Workers	PT	2
9/3/13	Large Workers	PT	3
9/3/13	Tray	PT	1.2
9/11/13	All Size Workers	PC	22
9/11/13	Large Workers	PC	33
9/11/13	Tray	PC	13.4
9/11/13	All Size Workers	PL	0
9/11/13	Large Workers	PL	1
9/11/13	Tray	PL	2.3
9/11/13	All Size Workers	PT	1
9/11/13	Large Workers	PT	0
9/11/13	Tray	PT	0.8
9/16/13	All Size Workers	PC	31
9/16/13	Large Workers	PC	22
9/16/13	Tray	PC	17.5
9/16/13	All Size Workers	PL	2
9/16/13	Large Workers	PL	1
9/16/13	Tray	PL	6.3
9/16/13	All Size Workers	PT	1
9/16/13	Large Workers	PT	0
9/16/13	Tray	PT	0.6
9/23/13	All Size Workers	PC	22
9/23/13	Large Workers	PC	30
9/23/13	Tray	PC	17.5
9/23/13	All Size Workers	PL	2
9/23/13	Large Workers	PL	0
9/23/13	Tray	PL	4.7
9/23/13	All Size Workers	PT	0
9/23/13	Large Workers	PT	1
9/23/13	Tray	PT	0.5
10/1/13	All Size Workers	PC	23
10/1/13	Large Workers	PC	42
10/1/13	Tray	PC	17.9
10/1/13	All Size Workers	PL	0

Date	Trap Type	Phorid Type	Count
10/1/13	Large Workers	PL	1
10/1/13	Tray	PL	8.9
10/1/13	All Size Workers	PT	1
10/1/13	Large Workers	PT	2
10/1/13	Tray	PT	0.7

Appendix 7. Parasitism rates of imported fire ants (IFA) by *Pseudacteon* spp. flies for each field location and for each date sampled.

Location	May 2013			June 2013			August 2013			October 2013		
	# Fly	# IFA	% Para.	# Fly	# IFA	% Para.	# Fly	# IFA	% Para.	# Fly	# IFA	% Para.
Auburn Beef Unit	7	18,750	0.038	15	18,750	0.08	12	18,750	0.064	17	18,750	0.091
Story Farm, Loachapoka	5	18,750	0.027	6	18,750	0.032	6	18,750	0.032	13	18,750	0.069
Silva Farm, Notasulga	8	18,750	0.043	10	18,750	0.053	17	18,750	0.091	17	18,750	0.091
Totals	20	56,250	0.035	31	56,250	0.055	35	56,250	0.062	47	56,250	0.084