Larval stages (instars) of the coffee leafminer *Leucoptera coffeella* (Guérin-Mèneville) (Lepidoptera: Lyonetiidae) and its synchronization with the parasitoid *Mirax insularis* Muesebeck (Hymenoptera: Braconidae) in Puerto Rico.

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ABSTRACT

The coffee leaf miner (CLM) *Leucoptera coffeella* (Lepidoptera: Lyonetiidae) is the main pest of coffee in the western central region of Puerto Rico. *Mirax insularis* (Hymenoptera: Braconidae) is one of the principal parasitoids of this pest present on the island. Three main objectives were carried out in this study: 1) to determine the number of instars of the CLM larvae under laboratory conditions, 2) to determine the number of instars in field collected samples and 3) to identify the instar (s) of *L. coffeella* preferred by *M. insularis* for its parasitation. Samples of leaves infested with CLM were obtained from the coffee growing zones in Puerto Rico. Colonies of *L. coffeella* were reared under laboratory conditions and the larval head capsule widths were measured across their widest point, and the results were plotted. Larvae from the field collected samples were measured and analyzed in the same way. Four larval instars were observed in both cases (laboratory and field).The preferred instar of *L. coffeella* by *M. insularis* was determined under laboratory conditions. Coffee plants were oviposited by *L. coffeella* during 48 hours, and when each instar was estimated to be formed, 150 parasitoids were introduced during 48 hours for its parasitization. The selection coefficient revealed that *M. insularis* parasitized 1st and 2nd instar with a 60% and 63% respectively. Instars 3rd and 4th were not parasitized.

RESUMEN

El minador de la hoja del café (MHC) *Leucoptera coffeella* (Leucoptera: Lyonetiidae) es la plaga principal de la planta de café en la región centro-oeste de Puerto Rico. *Mirax insularis* (Hymenoptera: Bracronidae) es uno de los principales parasitoides de esta plaga presente en la isla. Tres ensayos fueron realizados en este estudio: 1) determinar el numero de estadios larvales del MHC bajo condiciones de laboratorio 2) determinar el numero de estadios larvales del MHC en muestras colectadas del campo y 3) identificar el (los) estadio (s) larval (es) de *L. coffeella* preferido (s) por *M. insularis* para su parasitación. Muestras de hojas con minador del café fueron obtenidas desde las zonas de cultivo de café in Puerto Rico. Se criaron colonias de la especie *L. coffeella* bajo condiciones de laboratorio, se midió el ancho de su capsula cefálica a través de su punto más ancho y los resultados se graficaron. Larvas provenientes de muestras colectadas en campo se midieron y analizaron de igual manera. Se observaron cuatro estadios larvales en ambas condiciones (laboratorio y campo). El estadio larval preferido de *L. coffeella* por *M. insularis* se determinó bajo condiciones de laboratorio. Se ovipositaron plantas de café con *L. coffeella* durante 48 horas y cuando se estimó que cada estadio larval estaba formado, 150 parasitoides se introdujeron durante 48 horas para su parasitación. El coeficiente de selección reveló que *M. insularis* parasitó los instars 1^{er} y 2^{do} con un 60% y 63% respectivamente. Los estadios larvales 3^{ro} y 4^{to} no fueron parasitados.

To my husband, *Miguel*,

for you unconditional confidence love, patience and support.

To my parents,

because you guided me to be what I am.

"Gracias a la vida que me ha dado tanto.
me ha dado la marcha de mis pies cansados;
con ellos anduve ciudades y charcos,
playas y desiertos, montañas y llanos…."

Violeta Parra

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1 INTRODUCTION

In the West-Central region of Puerto Rico, coffee is the main agricultural crop. The annual coffee production for the years 2005-06 was $41.561 million, representing 13 percent of agricultural revenues for the island (Puerto Rico Department of Agriculture, 2006). However, one limiting factor for the coffee production is the coffee leafminer (CLM), *Leucoptera coffeella* (Lepidoptera: Lyonetiidae). The larva of this insect feeds on the mesophyll of the coffee leaves for three weeks, diminishing the photosynthetic activity and therefore, decreasing coffee production (Gallardo, 1987). The damage caused by the CLM consists of large brown spots on the leaves (Gallardo, 1987) that correspond to a mine, where they live and feed inside (Wolcott, 1947). Affected leaves soon drop off and, if the pest is not controlled, the photosynthetic activity can be reduced by 50% causing weight loss in the trunk, branches (70%) and roots (60%) (Monroig *et al*., 2002).

Pest control is mainly done with the insecticides Disyston 15G (disulfuton) and Temik 15G (aldicarb). Both are soil systemic and are applied twice a year (Monroig et al., 2002). Because of the problems associated with the use of chemical pesticides, e.g., environmental hazards, high cost, development of resistance, it is necessary to evaluate other control methods, less harmful and less expensive, such as biological control (Gallardo, 1992). *Mirax insularis* (Hymenoptera: Braconidae) is one of the principal parasitoids present in the coffee growth in Puerto Rico (Gallardo, 1987). This wasp was introduced in 1940 from Guadalupe to control CLM populations because of the high parasitization observed in its place of origin (65-85%) (Gallardo, 1992). This imported braconid is well distributed throughout all coffee regions of Puerto Rico (Gallardo, 1992).

Augmentation of the *M. insularis* population would be successful because this parasitoid is already well established throughout the coffee region in Puerto Rico. The implementation of this technique could eliminate all the costly and timely steps of foreign exploration and quarantine procedures needed (Gallardo, 1992). To establish an

augmentation program of *M. insularis* it is necessary to know the immature stages of the CLM to determine which of them (instar) is or are preferred by the parasitoid. Li *et al*., (2006) mentioned that for a successful synchronization between parasitoid and host, it is important to determine the stage most effectively parasitized. With this information females of the parasitoids could be released at the most effective time for best management in a program of biocontrol for the coffee leafminer.

The main objective of this investigation was to synchronize the parasitoid *Mirax insularis* with its host, *Leucoptera coffeella*, and to determine a strategic time for this. The specific objectives were:

- To determine the number of instars that the CLM has under laboratory conditions;
- To determine the number of instars that the CLM has from field collected samples in Puerto Rico;
- To identify the instar (s) of *L. coffeella* preferred by *M. insularis*, for its parasitation under laboratory conditions.

Whit this knowledge, the parasitoid can thus be mass-reared and liberated on selected coffee plantations throughout Puerto Rico. Results could have a great impact on promoting the use of *M. insularis* as a biocontrol agent (Gallardo, 1992). Success of this practice can decrease the use of systematic insecticides, resulting in economic and environmental benefits in Puerto Rico and other countries of the Caribbean Basin.

2 LITERATURE REVIEW

2.1. The coffee crop

2.1.1 Origin and distribution

Botanical evidence suggests that the coffee plant (*Coffea arabica* (L.) was originated in central Ethiopia and coffee drinking was introduced to Europe by the Arabs in the 17th century (Kimani *et al*., 2002). Today, the main areas of coffee production around the world are: South and Central America, the Caribbean, Africa, Arabian Peninsular, India, Indonesia and New Guinea (USDA, 2006).

2.1.2 Economic importance

Coffee consumption has been increasing worldwide and is consumed by an estimated of one third of the world's population. Moreover, coffee is being one of the major global commodities (Kimani *et al*., 2002). Today, Brazil is the largest coffee producer and exporter. Colombia, Vietnam, Indonesia and Mexico are other important producer and exporter (USDA, 2006).

Coffea arabica, was brought to the Caribbean islands by the French, and was introduced to Puerto Rico in 1736 from Martinique and Haiti. In 1755, coffee was established as a commercial crop in the island (Mondoñedo, 1957). Van Zwaluwenburg (1917) mentioned that coffee has been cultivated for 150 years in Puerto Rico; so today in 2007 it is possible to affirm that coffee has been cultivated in the island for almost 250 years.

Coffee is considered as one of the main agricultural products of Puerto Rico. Until the year 2002, this crop occupied the second most important place among crop commodities in the country (Monroig *et al*., 2002). During the period 2004/05 the production was $ 34,000,000, representing 13% of the total crop production (Puerto Rico Department of Agriculture, 2006). For 2002, the yearly production in Puerto Rico was estimated in 65,000 acres and nearly 95% of the total production was harvested. The coffee production area is composed of 22 municipalities, located mainly along the western central part of the island, where the terrain is hilly, and the climate is humid with an average rainfall of 1905 mm (Alvarado, 2002). Ninety seven percent of the coffee production in Puerto Rico is destined mainly for local consumption, and the other 3% is exported (Alvarado, 2002). However, production is seriously affected by pests and diseases that affect directly and indirectly the crop (Monroig *et al*., 2002).

2.2 Insect pests

2.2.1 Coffee pests worldwide.

In general, over 900 species of insects are considerered coffee pests around the coffee production areas. However, only some of them are economically damaging (Kimani, 2002). The main pests worldwide are: *Leucoptera coffeella* (Guérin-Mèneville), *Coccus viridis* (Green), *Saissetia hemisphaerica* (Targioni), *Planococcus citri* (Risso), *Toxoptera aurantii* (Boyer de Fonscolombe), *Hypothenemus hampei* (Ferrari), and *Solenopsis invicta* (Buren) (Monroig *et al*., 2002).

2.2.2 Coffee pests in Puerto Rico

According to Monroig (2004), the insects that mainly attack the coffee crop in Puerto Rico are: L*eucoptera coffeella*, *Lachnopus coffeae* (Marshall), *Apate monacha* (Fabricius), *Psychonoctua personalis* (Grote), *Xilosandrus morigerus* (Blandford), and *Coccus viridis* (Green) being *L. coffeella* the mainly present.

At this moment, *L. coffeella* is a key pest in all countries where the coffee is cultivated. In Puerto Rico the presence of this leafminer dates back to 1921 (Wolcott, 1947), and today about 70% of coffee plantations are affected in a given year, although 100% of the crop is at risk (Monroig *et al*., 2002).

2.2.3 Coffee Leafminer

The CLM, a caterpillar of the *Leucoptera* genus, it is a micro-Lepidopteran of the Lyonetiidae family with crepuscular nocturnal habits (Guerreiro, 2006), and described by Guérin-Mèneville and Perrottet in 1842 from specimens collected from Guadaloupe and Martinique (Cardenas and Benavides, 1990). Guérin-Mèneville and Perrottet placed the CLM in the genus *Elachista*, but later it was referred as *Cemiostoma* by Station (Station, 1861). Through a misidentification, the common *Leucoptera meyricki* (Ghesq) found in Africa, was referred to as *L. coffeella* in nearly all the literature until 1958, when Bradley (1958) eliminated this confusion by distinguishing it from *L. coffeella*. A synonymy of *Perileucoptera* has been proposed by Silvestri in 1943, but is only used in Brazil (Souza, 1979).

The larvae of the CLM penetrate the coffee leaf and eat from the mesophyll for about three weeks. The larva feeds exclusively from coffee leaves (Ramiro *et al*., 2004). If not controlled it may reduce photosynthetic activity by 50% and cause marked weight loss of the trunk, branches and roots (Monroig *et al*., 2002). After eclosion, the larvae perforate the upper epidermis of the leaf and penetrate the mesophyll, eating the palisade parenchyma cells (Konnorova and De La Vega, 1985). The larvae burrow

inside the leaf and eat on the tissue between the leaf surfaces resulting in several small mines that can grow together (Kimani *et al*., 2002). The lesions that form between the epidermises, also called mines, galleries or tunnels, have irregular margins and pale yellow color which later become brownish (Konnorova and De La Vega, 1985). The damage in the plant is greater in the upper third part of the plant canopy and the production losses are directly related to the intensity of attack and to the period in which this occurs (Guerreiro, 2006).

The intensity of the damage caused by this pest depends on several aspects such as cultural practices, seasons and crop area (Almeida, 1973), climatic conditions (long periods of drought associated to high temperature) and, above all, the inadequate use of chemical control agents (Costa *et al*., 2005). For example, in some parts of Africa, the CLM is a destructive pest as a result of the heavy use of pesticides which has eliminated many of the pest's natural enemies in coffee groves (Kimani *et al*., 2002).

The necrotized leaf surface (Figure 1) caused by the CLM reduces the photosynthetic activity of the leaves (Cibes and Pérez, 1958), affecting the flux of water, minerals and organic matter (Konnorova and De La Vega, 1985). However, the loss in production is mainly due to the leaf loss (Crowe, 1964) provoked by increased levels of ethylene (Souza *et al*., 1998) mainly when the lesions are near the petiole (Notley, 1956).

In Puerto Rico all of the coffee cultivated area is at risk of CLM attack, and up to 70% is affected every year (Monroig *et al*., 2002). In Brazil, 21.6% yield loss was reported when 46.24% of the coffee leaves were damaged by CLM (Nantes and Parra, 1977a). In the same study, 24%, 50% and 75% of the leaf surface reduction was observed during the dry season resulting in coffee production losses of 9.14%, 23.53% and 87.24%, respectively (Nantes and Parra, 1977a).

Figure 1. CLM Damaged leaves.

2.3 The coffee leafminer complex

The CLM-complex includes the species *Leucoptera meyricki* (Ghesq), *L. coma* (Ghesq), *L. caffeina* (Wshbn) and *L. coffeella* (Guérin-Mèneville). The first three are found exclusively in Africa. *Leucoptera meyricki* is the most common and has been reported in the Ivory Coast, Angola, Congo, East Africa, Ethiopia and Madagascar. *Leucoptera coffeella* is also confined to South and Central America, and the West Indies (Hill, 1975). According to Le Pelley (1968), the four species of the genus *Leucoptera* are distributed as follows: *L. coffeella* (South and Central America, and the Caribbean), *L. meyricki* (Africa), *L. caffeina* (Angola, Zaire, Kenia and Tazania*),* and *L. coma* (Zaire and Uganda).

Leucoptera meyricki and *Leucoptera caffeina* are the most important species, considering to *L*. *meyricki* as one of the dominant species. However, this has not always been the case. In the 1930's *L. caffeina* was considered to be dominant in shaded

coffee and *L*. *meyricki* only under unshared conditions (Bigger and Tapley, 1969). Crowe (1964) mentioned that factors as adoption of mulching, increased use of copper fungicides, the use of DDT, and later the use of dieldrin have been contributed to increase this insect. In Africa, all coffee species appear to be attacked by different species of CLM, but *L caffeina* only appears to thrive on *Coffea arabica*. In Kilimanjaro (Tanzania-Africa), *L caffeina* has been recorded from *C. canephora*, *C. arabica* var. erecta, *C. canephora* var. robusta, *C. congenesis*, *C. eugenioides*, *C. arabica* var. maragogype, *C. canephora* var. Quillou and *C. excelsa* (Notley, 1956).

Leucoptera coffeella is the major pest of *Coffea arabica* and *Coffea canephora* in Brazil and others countries in South America (Medina *et al*., 1977). The coffee leafminer is considered as a pest in México and Colombia (Cardenas and Benavides, 1990), Guatemala and Venezuela (Cabrera, 1989), Honduras (Nantes and Parra, 1977b), El Salvador (Hanania, 1989), Nicaragua (Flores, 1981), Perú (Enríquez *et al*., 1975) and Brazil where it was introduced in 1921 (Parra, 1985). In Puerto Rico this pest was informed by Wolcott in 1947 (Wolcott, 1947).

It is possible to find different species of *Leucoptera* affecting different cultivars of coffee and other crops of the family Rubiaceae. Additionally, in a taxonomic study made by Mey (1994), 20 species of the genus *Leucoptera* were informed infecting 65 host species, belonging to six plant families: *Betulaceae, Hypericacea and Salicaceae, Rosaceae, Fabaceae and Aceraceae.*

Mines of *L. caffeina* can be easily be distinguished from *L. coffeella* by the ovipositional arrangement of the eggs. *L. caffeina*'s eggs are laid in a row on the upper surface of the leaf, and very occasionally, more than one row may be found. In contrast, the eggs of *L. coffeella* are scattered in small groups on the upper surface of the leaf, not adjacent to each other, and are laid randomly (Notley, 1956). Crowe (1964) found that eggs of *L. meyricki* are laid on leaves without shade and very close to each other, unlike *L. caffeina* that prefers leaves with shade and are laid irregular groups of eggs.

2.4 *Leucoptera coffeella*

2.4.1 Moth description

The adult of the coffee leafminer is a moth of about 3.5 mm long with a wingspan of 6.5 mm. They have a silvery white scales covering the head and face, the body below, the upper side of the front wings and legs, with the exception of the tips of the first, second and fourth tarsi (Figure 2). When the moth is not in flight, the wings are folded close to the body. The front wings are longer in proportion to their breadth and in the upper side of each, at the extremity of the inner edge there is a large steel-blue or black spot (Cook and Horne 1905, Guerreiro, 2006 and Pickman, 1872).

From the front of the head this moth projects a spreading tuft of silvery hairs, which forming a velvety eye-cap as large as the eyes. It has filiform antennae that are about three-fourths long as the front wings and their basal joints are thickly clothed with silvery hairs (Pickman, 1872).

Figure 2. Adult of *Leucoptera coffeella* (magnification 40x)

2.4.2 Life cycle

Leucoptera coffeella is an holometabolous insect (Souza *et al*., 1998). Its life cycle has a duration from 7 up to 42 days. Pickman (1872) indicates a life cycle of less than 14 days. Wolcott (1921) obteined life cycle duration from seven to 21 days, Hernandez (1972) from 14 to 42 days, and Nantes and Parra (1977b) from 25 to 34 days. Similarly, research on the duration (days) of the different stages shows that duration appear to be influenced by different climatic conditions (Da Fonseca,1944; Katiyar and Ferrer, 1968, and Pruna and Licor 1973).

2.4.3 Egg stage

The egg is oval, pearl-colored, and it has a depression at the center (Wolcott, 1921). The eggs are scattered in small groups on the upper surface of the leaf, are not adjacent to each other, and are laid at random (Notley, 1956).

The oviposition is strongly influenced by temperature and air humidity, which is affirmed by Katiyar and Ferrer (1968), and demonstrated in studies made by Parra (1985). These authors proved that females reared in artificial conditions at 27^{o}C with5 nearly 100% relative humidity, laid more than 60 eggs, especially between the 2nd and 6th day of adult life. The oviposition takes place during the night. Unmated females may lay a few eggs, but these do not hatch (Notley, 1948).

On the other hand, Konnorova (1986) concluded that at 27^{o}C, the egg eclosion occurred in two days, and higher or lower temperatures delayed the eclosion period. The duration of the egg can be over 20 days (Katiyar and Ferrer, 1968), and can be reduced from four to six days under the optimum conditions of 27^{0}C, with almost 100% HR and 14h of photoperiod (Parra, 1985).

2.4.4 Larval stage

Close to the time of eclosion, the egg turns brown (Figure 3a) due to the excrement of the larva (Speer, 1949); the eggs turn pale brown about one day before hatching. From two to four days after, the mines made by each larva of a group (which at first are roughly circular), join up to form a common mine (Figure 3b) (Notley, 1948).

The larva may live from 9 to 42 days inside the leaf depending on the temperature. After that, the larva leaves the mine through a semicircular slit in the upper surface, from where more that one larva may leave (Speer, 1949). According to Notley (1948), the duration of the larval stage is variable. Thus, for example, a larva at 24^{o}C fully develops at 13 days and, at 19 ^{o}C the larvae takes 24 days to develop. On the other hand, higher temperatures, and high relative humidity shorten the larval phase. Parra (1985) demonstrated that the larval phase on leaves of *C. arabica* cv Mundo Novo varied between 7 to 11 days at 27 ^{o}C and 30 ^{o}C, respectively.

Notley (1948, 1956) found that CLM has four larval instars, which could be in the same mine at the same time. There is no evidence of cannibalism between larvae in the same mine, even when mines containing young and old larvae coalesce (Notley, 1948). The damage caused by this species was reported as with an average area of leaf-mine per larva of 50 mm^2 (Notley, 1948).

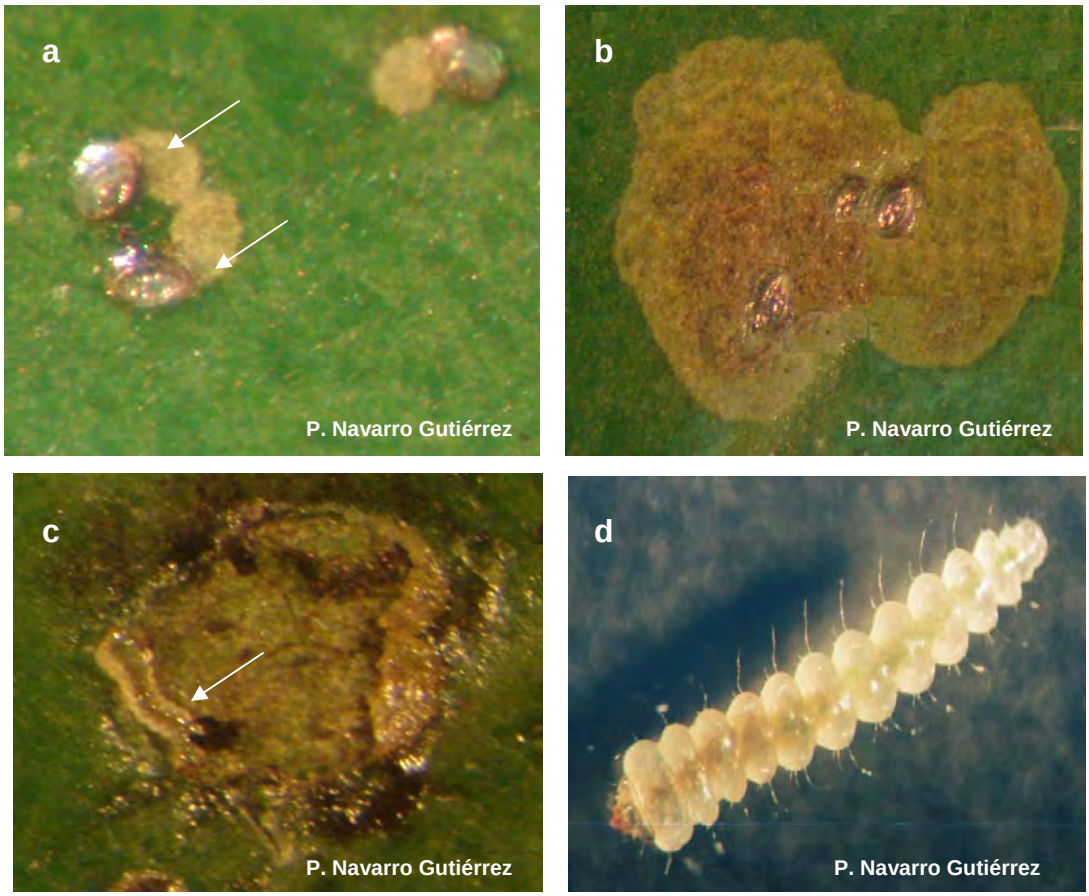

Figure 3. Development of *Leucoptera coffeella*'s larva. a) hatching eggs, b) common mine, c) open mine with *L. coffeella*'s larva inside d) larva. (magnification 40x).

2.4.5 Pupal stage

The last larval instar leaves the mine and moves around for a couple of hours looking for a place on the surface of the leaf where to make its cocoon (Wolcott, 1921), which normally is at the axial part of the leaves (Guerreiro, 2006). First, the larva spins silk threads in form of "X" (Figure 4), simulating a spider web, on a depression of the leaf. Underneath of that, the larva spins a white cocoon and transforms into a pupa by expelling its old skin. The skin is expelled through an opening at the cocoon's caudal end (Wolcott, 1921).

The prepupal stage lasts from 30 to 36 hours, from the completion of the cocoon until the shedding of the larval skin (Notley, 1948). The duration of the pupal phase varies with the temperatures. For example, Parra (1985) found that with temperatures varying from 20^{o}C to 35 ^{o}C large variations were found in the duration of the pupal phase. At higher temperatures the duration is reduced. Adults emerged after an average of 14 days at 20^{o}C, and of 3.6 days at 35 ^{o}C. Temperatures between 27 ^{o}C and 30 ^{o}C were found to be favorable for development, especially for the pupal phase. Under these conditions, about 95% reach the adult phase (Parra, 1985). According to Notley (1948), the pupal phase is shorter in females than in males.

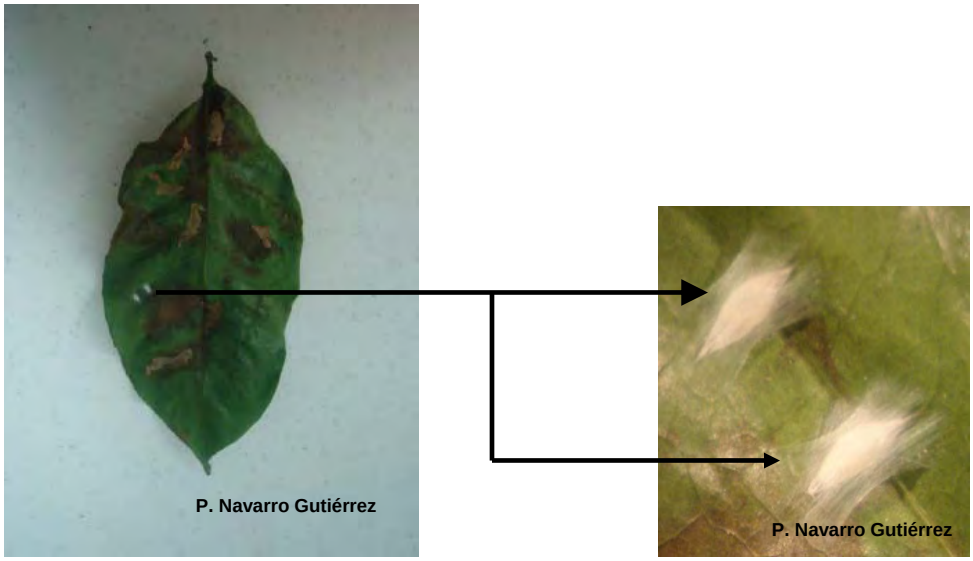

Figure 4. Pupal phase of *Leucoptera coffeella* (magnification 40x).

2.4.6 Adult stage

Guerreiro (2006) states that, although the most important damage caused by the larva, it is necessary to learn about the adult phase to control it. Aspects like female fecundity, host preference, and susceptibility to certain chemicals are intrinsic to this phase. General aspects about the adult and its life cycle are, for example, the total life-cycle, from egg to emergence of adult, which is 27-30 days at 24 ^{o}C, and 45-50 days at 19.5 ^{o}C. Females fed only with water live a shorter time and laid fewer eggs than females fed with sugar solution (Notley, 1948). The CLM has a 1:1 sex ratio (Speer, 1949; Parra, 1985).

2.5 Determination of the larval instars through the Head Capsule Width Parameter

Head capsule width (Figure 5) is a parameter commonly used for determination of larval instars. This information answers to questions of interest like, which instar is prey of a determinate predator?, which instar is parasitized by determinate parasitoids?, and which are more susceptible to the infection of determinate pathogens? (Schmidt *et al*., 1977).

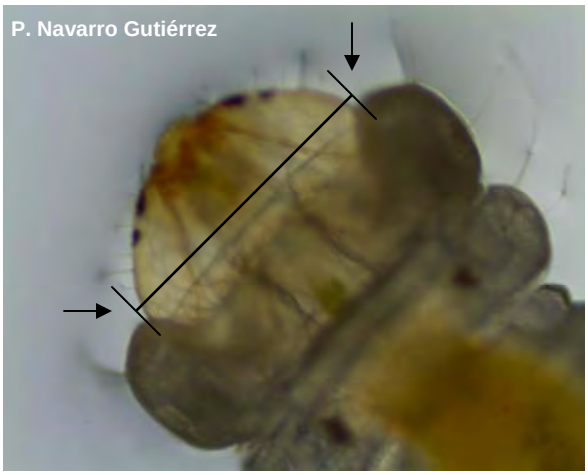

Figure 5. Head Capsule Width measure (µm).

This methodology consist in measure the larvae's cephalic capsules in their wider section by means a micrometer. Thus, the total number of peaks represented in a head capsule size frequency distribution, shows the total number of instars in a species (Schmidt *et al*., 1977). It is assumed that each peak represents an instar and the distribution of the head capsules sizes in each instar has a normal distribution (Caltagirone et al., 1983). However, when it is necessary to find the number of instars of an insect, it is sometimes difficult to decide if one of them has been overlapped or not (Crosby, 1973). Several methodologies had been studied to minimize this overlap being the Got's formula, about discrimination limits, one of the most used.

2.6 Biological Control

According to DeBach and Hagen (1964) the concept of biological control involves "… the action of parasites, predators, or pathogens in maintaining another organism population density at lower average than would occur in their absence".

The conditions that prevail in Puerto Rican coffee plantations increase the probabilities of success of a biocontrol program against the CLM (Gallardo, 1992). These conditions are: First, the coffee plant is a perennial tree growing in conditions that allow a constant succession of CLM generations (Lloyd, 1960; Hall and Ehler, 1979). Second, the CLM is an indirect pest of coffee. Indirect pests are more appropriate targets for biocontrol (Coppel and Mertins, 1977). Third, that Puerto Rico is a tropical island with a mild warm climate, and under island conditions a high degree of success can be expected because of the diversity with many ecological niches available (MacArthur and Wilson, 1967; Greathead, 1971).

2.6.1 Methods

There are three different methods of biological control: introduction, conservation and augmentation. Introduction and conservation have been used by Speight *et al*., (1999) and Ruberson (1999), respectively. Augmentation can be either accomplished by periodic colonization or inoculation, by development of adapted strains by artificial selection or by inundation (De Bach and Hagen, 1964). One such method includes the increase of the parasitoid population of the parasitoid directly through insectary propagation and release into the environment (King *et al*., 1981; Luck *et al*., 1999). The augmentation of *M. insularis* using that method seems to be the most feasible and inexpensive way to start a biocontrol project of the CLM in Puerto Rico (Gallardo, 1992).

2.6.2 Biological control of *L. coffeella*

In Brazil during the 1940's, CLM was controlled by natural biological control and cultural techniques (Le Pelley, 1968). After the indiscriminate use of insecticides, an ecological imbalance occurred producing a collapse of natural CLM controls (Villacorta and Wilson, 1994). In the search of new alternatives of biological control, species of the families Vespidae and Chrysopidae were studied (Gravena, 1984; Carvalho *et al*., 1994).

In Brazil, Souza (1979) reports the predators (Hymenoptera: vespidae): *Protonrctarina sylveirae* (Saussure), *Polybia scutellaris* (White), *Brachygastra lecheguana* (Latreille), *Polistes sp*, *Apoica pallens* (Fab) and *Polistes versicolor* (versicolor). In Colombia, Santis (1983) reports the parasitoids (Hymenoptera: Eulophidae): *Zagrammosoma sp.* and *Pnigalio sarasoli*. Also in Colombia Cardenas (1991) reported the predators *Polistes spp*. (Hymenoptera: vespidae), *Polybia sp.* (Hymenoptera: vespidae), *and Chrysopa sp.* (Neuroptera:Chrysopidae) attacking CLM. In Perú, the most important natural enemies (Hymenoptera: vespidae) were: *Polybia*

juruana (Ihering), *P. reyecta* forma belizansis, *Polistes peruviana* (Bequaert), *P. versicolor* (Olivier) (Enriquez *et al*., 1975).

First reports of CLM natural enemies in Puerto Rico were given by Van Zwaluwenburg (1917). Later, Wolcott (1947) found 10 parasitoid species (Hymenoptera: Eulophidae) in order of abundance: *Closterocerus leucopus* (Ashmead), *Chrysocharis lividus* (Ashmead), *Horismenus cupreus* (Ashmead), *Zagrammosoma* sp. (Nov). *Closterocerus* sp. *Cirrispiloideus* sp. (Nov), *Darastenus* sp., *Tetrastichus* sp. (nov), and *Microbracon spp.* Also was reported by the same author *Telenomus sp*. (Hymenoptera: Scelionidae).

A survey conducted during 1985-1986 by Gallardo (1987), concluded that one of the principal parasitoids was *Mirax insularis* Muesebeck (Figure 6), which was parasitizing 33% of larvae. Later surveys have included the search for natural enemies of egg and larval stages of the CLM (Gallardo, 1992). One of these surveys was conducted by Gonzalez (1996), who did not find the presence of parasitoids or predators of the egg stage.

Mirax insularis is considered one of the most promising natural enemies of CLM in coffee plantations in Puerto Rico. This braconid was introduced from Guadaloupe and released in coffee plantations in Lares and Quebradillas before 1940 (Gallardo, 1987). *M. insularis*, is a koinobiont parasitoid. The length of its life cycle in Puerto Rico was described by Leon (1997). However, information about synchronization between the host and parasitoid life cycle has not been reported.

The genus *Mirax* was first described by Haliday in 1834:”eyes somewhat villose, abdomen showing 7 segments, 6 beneath smooth and shining. Radius of the fore wing hardly inchoate .The antennae present 12 segments and no occipital carina is present (Valeiro, 2007).

Figure 6. *Mirax insularis.*

3. Materials and Methods

3.1. Origin of the samples.

Samples of coffee leaves (*C. arabica*) with mines were obtained from Yauco and Adjuntas. Both zones are located in the mountainous area of Puerto Rico Farms. In these areas where collections were made no chemicals had been applied for at lest nine month. Thus it was presumed that CLM population in these areas was minimally manipulated.

Nursery coffee plants (*C. arabica* cultivar Limaní) without CLM were obtained from Adjuntas Experimental Station. These coffee plants were grown with no pesticide applications. Coffee plants with 10 to15 leaves were selected for best handling.

3.1.1 Characterization of the selected coffee growing areas.

Collection areas have an elevation of 470 meters (Yauco) and 580 meters (Adjuntas) above sea level, with an annual average of precipitation of 74.5 in (1893 mm). The rainy season includes the months of May, August, September and October, and the dry season the months of December, June, February and March. The highest and lowest annual averages are 82^0 F (28^0C) and 50^0 F (10^0C), respectively (Adjuntas Experimental Sub-Station, 2007).

3.2 Host's and parasitoid's colonies.

To develop the objectives of this work it was necessary to establish a CLM's colony and of its parasitoid under laboratory conditions. Both methodologies are presented below:

3.2.1 Colony of the host (*L. coffeella).*

3.2.1.1 Formation of the colony.

Coffee leafminer colonies were initiated from moths collected from field infested coffee leaves. Approximately 1000 leaves were collected weekly during the dry season. Mined leaves containing eggs, larvae and pupae were collected and transported to the Biological Control Laboratory (BCL), at the University of Puerto Rico at Mayaguez, in plastics bags (30cmx20cm) inside a cooler. In the BCL the mined leaves were maintained inside a plastic bug dorm (BD) (insect rearing tent 60x60x60 cm^3), with a high humidity level allowed to maintaining their leaf turgency for two to three weeks.

After this period, pupae were collected adhered to the leaf and placed in Petri dishes (15 x 5mm) (Figure 7). Petri dishes contained wet cotton (in distilled water) and filter paper, in order to maintain the turgency of the piece of leaf with the pupa.

Figure 7. Individual pupa in a Petri dish

3. 2.1.2 Rearing of the CLM.

The coffee plants were cleaned with distilled water to minimize the presence of any insect or pathogens that could be present. The coffee plants were placed inside a BD (Figure 8) where CLM were released daily as moths were emerging from the Petri dishes, for oviposition. Moths were maintained inside a BD at 80^0F (27^0C), 70% RH (by means a hygrometer) and a photoperiod of 12L: 12D (by means a timer).

Figure 8. Bug dorm to oviposition of *Leucoptera coffeella*

Adults were fed with 10% sucrose solution. According to Nantes and Parra (1977b) this solution increases the adult's longevity and number and viability of the eggs. For feeding, small vials (30 ml) with paper towel soaked in the sucrose solution (Figure 9) were deposited inside each BD. The contact of the insect with the paper towel allowed the insect to feed. Sucrose solution and paper towel were replaced daily to avoid the food fermentation.

Figure 9. Vial with sucrose solution

3.2.2 Colony of the parasitoid (*M. insularis*).

3.2.2.1 Formation of the colony.

Leaves were collected from coffee producing areas (Yauco and Adjuntas Farms) where the presence of *M. insularis* was previously known and identified. Leaves were transported and manipulated as described before. Pupae were extracted and put individually in Petri dishes (Figure 7). Once the parasitoids emerged they were released inside a BD with CLM infested plants.

3.2.2.2 Rearing of the parasitoid.

Once the parasitoid's colony was established in the BD, addition of new parasitoids were made by two ways: 1) adding parasitoids that emerged daily from the Petri dishes, 2) adding parasitoids that emerged from nursery plant previously parasitized by *M. insularis*.

Parasitoids were fed with 10% sucrose solution and were maintained inside of the BD at 80^0F (27^0C), 70% RH and a photoperiod of 12:12. The sucrose solution was distributed in a thin film on the BD's interior. Parasitoids were fed twice a day, each time cleaning the BD with paper towel soaked in distilled water. This was done to avoid the fermentation.

3.3. Experimental trials.

3.3.1 Determination of larval instars of the CLM under artificial laboratory conditions.

Six coffee plants were introduced inside a BD and 200 moths of CLM were release for oviposition. The moths were maintained inside the BD for 48 hours at 80^0F (27^0C), 70 % HR and 12:12 h of photoperiod. After that, moths were removed and the presence of eggs on the leaves was confirmed (day 0).

Starting on day 0, and the following days until the pupae began to be formed, five eggs were randomly removed from the coffee plants. The CLM eggs were slide mounted in euparal mounting medium. Embryo formation was determined observing the change in the egg's color from hyaline to brown, while still holding turgency. Non turgent eggs had no embryos. This observation was made by means of a stereoscope with a magnification of 40x. If the embryo was not formed (Figure 10a), data was recorded as

zero. In contrast, when the embryo was observed and the larva inside of egg was visible (Figure 10b), the larva's HCW was measured using a micrometer.

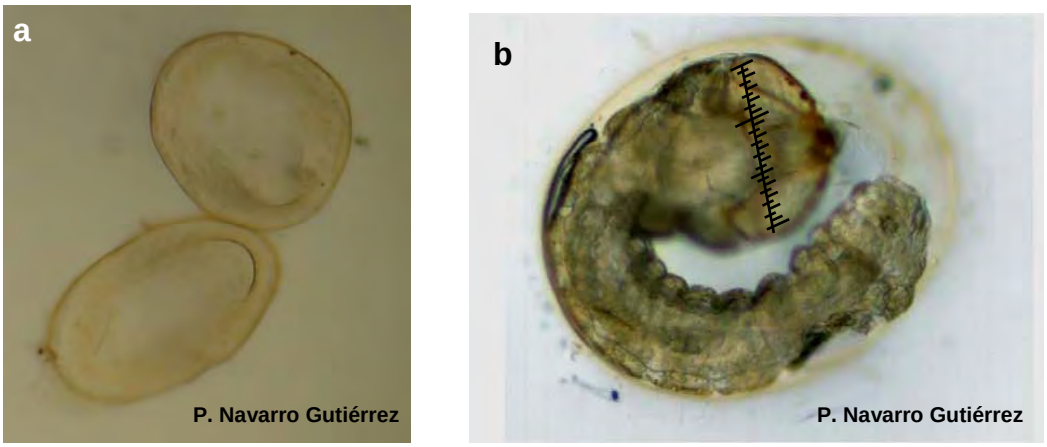

Figure 10. *Leucoptera coffeella*'s egg. a) without embryo, b) larva inside of egg

More than one larval instar can be found in the same mine at the same time (Notley, 1948). Additionally, as long as the larvae are inside the mine, was not possible to measure the HCW of the same larva throughout the time. Thus, when the larvae hatched from eggs (colored brown), five larvae were randomly removed from any mine in any of the six plants. The larvae collected were submerged in ethanol 70% for 30 minutes. After that, larvae were washed in distilled water and slide mounted in euparal mounting medium. This procedure was repeated until the larval cycle (inside of the mine) was completed and the pupae began to be formed. Measure of the HCW of each larva (1, 2…5) was made in its wider point by means of micrometer. The last head capsule measure to be considered was the one eliminated in the cocoon's caudal end.

Frequency distribution was determined by means of the statistics program INFOSTAT (2004). Head capsule widths were assumed normally to be distributed in peaks, and each peak represented an instar (Caltagirone *et al*., 1983; McClellan and Logan 1994). To determine the duration of each instar a smooth curve was interpolated (α=0.2) through a locally weighted regression (LOESS). Data was separated by clusters

and parameters such as average, variance, standard deviation, lower and upper ranges, and growth rates (according to Dyar, 1890) were calculated. To determinate overlap between neighboring adjacent instars, a discrimination method using limits was calculated. The discrimination method based on a probabilistic model of head capsule widths in successive instars proposed by Got (1988) and by Villa and Catalan (2004). This method consists in finding the value which minimizes the sum of the two probabilities of misclassifying as the discrimination point between two contiguous instars (limit). For the determination of this limit the follow equation was used:

$$\text{Limit}_{i,i+1} = \frac{\mu_i \sigma_{i+1} + \mu_{i+1} \sigma_i}{\sigma_{i+1} + \sigma_i}$$

Where:

i : instar number 1, 2, …

μ : average of HCW to larvae in a specific instar.

σ : standard deviation of HCW to larvae in a specific instar.

3.3.2 Determination of the number of larval stages (instars) of field collected CLM in Puerto Rico.

Two hundred coffee leaves infested with the CLM were randomly collected from the field. The leaves were collected for a period of four weeks in January and four weeks in August for the years 2006 and 2007. These months were selected because they correspond to yearly CLM's population peaks. The population of the CLM starts increasing from April through July, presenting the greatest abundance in August. From September to November CLM populations decrease (Gallardo, 2006).

Mined leaves were brought to the BCL, where larvae were extracted from each mine. The number of sampled larvae was determined with a 95% level of confidence through the sample size procedure given by INFOSTAT (2004). Larvae were removed from the mines with the help of a stereoscope, including the measure of the head capsule eliminated by the larva in its last molt, which is usually found close to the cocoon's caudal end. HCW measurements were conducted as previously described. The parameters as frequency, average, variance, standard deviation, lower and upper ranges, and growth rates were determined as was described before (section 3.3.1).

Finally, morphological characteristics of the larva were identified by differentiating the most important structures present in the buccal apparatus, setae, and prolegs. The critical point drying methodology was developed for sample preparation for electronic microscopy technique (SEM), which consists in a scanning electron microscope capable of producing high-resolution images of a sample (larvae). For this, larvae were submerged for 5h in 2.5% glutaraldehyde. After that, larvae were washed in distilled water and then were gradually dehydrated for 15 minutes in ethanol (30%, 35%, 40%, 45%, 60%, and 70% respectively). Finally larvae were mounted on aluminum stubs with colloidal silver, sputtered with 10 nm Au/Pd, and examined with a Zeiss scanning electron microscope.

3.3.3 Identification of instar(s) of *L. coffeella* preferred by *Mirax insularis*, for its parasitization under laboratory conditions.

3.3.3.1 Relation between the numbers of *L. coffeella* versus *M. insularis* from field collected samples.

Coffee farms growth areas where *M. insularis* was observed (flying) in the field were identified and selected for this study. From these areas, two hundred coffee leaves containing eggs, larvae and pupae of the CLM were collected twice during January 2007. The relation between the number of CLM and parasitoids present in the sample was determined.

Larvae were allowed to complete development to pupae, and handled as described before. These leaves were observed daily to verify the emergence of the insects in study (*L. coffeella* or *M. insularis*), and thus to differentiate the species. The verification of the hymenopterous species was made by means of stereoscope.

3.3.3.2 Determination of the sex ratio.

The relationship between the number of male and female in both species (*L. coffeella* or *M. insularis*) was determined. Identification was made by means of a stereoscope as before. Moth sex was determined from the shape of the two last abdominal segments (Nantes and Parra 1977b). Females of *M. insularis* were differentiated from males by means of its ovipositor.

3.3.3.3 Identification of the preferred instar (s).

Inside a BD, six coffee plants were oviposited by two hundred moths of *L. coffeella*. The laboratory conditions were 80^0F (27^0C), 70% HR and 12:12 h of photoperiod. Moths were maintained inside the BD for 48 hours for oviposition and later were removed. The presence of eggs was confirmed (day 0) and then the larval cycle of the leaf miner followed its normal course.

With the information obtained from objective 1, each instar was distinguished by the age of the larva in days. This way, each BD contained only one instar (I, II, III…). Four replicates of each instar were performed. Each replicates consisted of BD with six coffee plants. When each instar was estimated to be formed, 150 parasitoids were introduced during 48 hours for oviposition. The relationship between male and female of introduced parasitoids was determined according to results obtained from observations previously mentioned. After 48 hours, the parasitoids were removed and parasitized larvae allowed their normal cycle.

When the cycle of each instar ended and the pupae were formed, the total number of pupae was counted. After that, pupae were cut from the leaf, and put individually in a Petri dish. Each Petri dish contained wet cotton and filter paper in order to maintain the turgency of the leaf where the pupa was adhered. When adults emerged, the number of parasitoids and leafminers were counted to determine a numerical relation. Petri dishes were used to maintain the humidity and to observe if superparasitism was present; consequently, if more than one parasitoid emerged from a pupa, it was considered as superparasitism. The dissection of larvae was not contemplated as a technique to determinate superparasitism because it affects the final number of host (pupa) and parasitoids (inside) in the sample.

The formula used to complete the percentage of parasitism and the selection coefficient was presented by Cook (1978) and Li *et al*. (2006). This observation was made for each instar and the host age preference for the parasitoid was determined according to the greatest percent of emergency of the parasitoid. The experimental design consisted in a randomized complete block design (RCBD), with 4 replicates and was analyzed with ANOVA analysis and Tukey's multiple range test (INFOSTAT, 2004).

4 RESULTS

4.1 Determination of larval instars of the CLM under artificial laboratory conditions.

4.1.1 Larval instar number

A histogram of head capsule widths of laboratory reared larvae is presented in Fig.11. The collected data ranged from 13 µm to 44 µm, and the duration of the larva's cycle under laboratory conditions was of 13 days (Figure 12). The frequency distribution analysis of HCW shows four distinguishable larval instars. Each peak within the distribution was assumed to correspond to an individual instar. First and fourth instars presented the largest number of larvae in the analyzed data. In addition, in the same figure it is possible to observe that the four instars (1^{st} and 2^{nd}, 2^{nd} and 3^{rd}, and 3^{rd} and 4^{th}) are somewhat overlapping. This means, that each larval instar may have cephalic capsules that could belong to the adjacent one.

In order to classify each instar in relation to time (days), a smooth curve was interpolated, using locally weighted regression (Figure 12). This curve shows the distribution of predicted values (LOWESS) of HCW per instar. Four distinct peaks, as in a frequency distribution before analyzed, were observed. According to Figure 12 and Table 1, the duration of 1^{st} instar was three days, 2^{nd} and 3^{rd} instars four days and 4^{th} instar 2 days. The data of each instar was used to determine descriptive parameters such as mean, variance, standard deviation and, lower and upper ranges (Appendix 1). The second instar presented the highest variance and standard deviation, and 4^{th} instar the lowest. The second instar also presented the wider range of HCW between lower and upper values (µm).

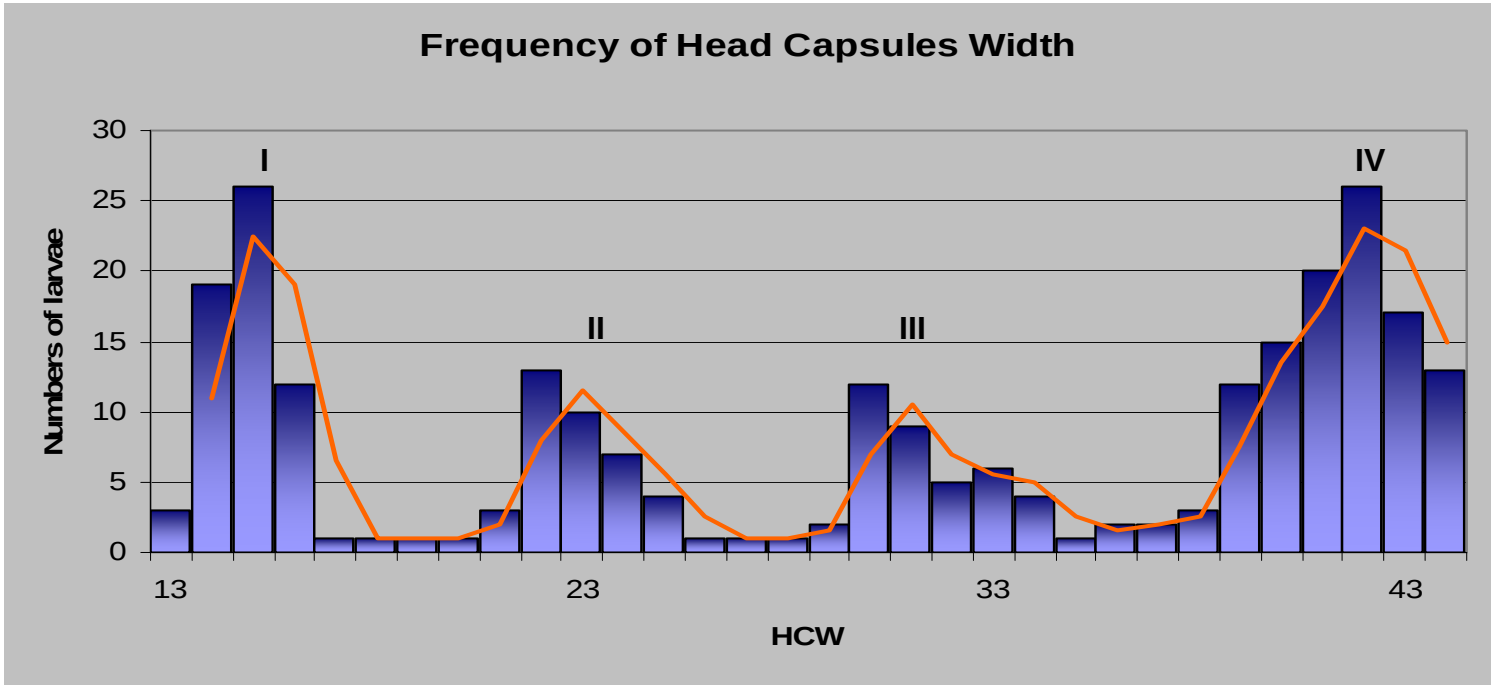

Figure 11. Frequency distribution of head capsule widths of *L. coffeella* under laboratory conditions.

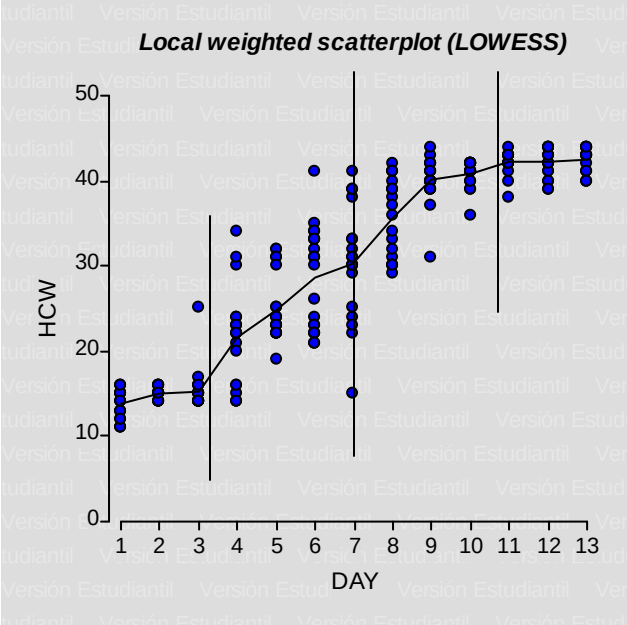

Figure 12. Distribution of measures of head capsule widths of *L. coffeella* during the life cycle of the larva under laboratory conditions.

Table 1. The estimated time period (days) of head capsule width and parameters[a] of normal distribution functions.

Instar	Period according Predicted values (days)	m[a] (μm)	σ^2 $(\mu m)^2$	SD[a] (μm)	Minimun (μm)	Maximun (μm)
I	1 – 3	13.9	17.3	4.3	11	25
II	4 – 7	26.5	41.0	6.4	14	41
III	8 – 11	38.7	12.8	3.5	30	43
IV	12 – 13	42.2	2.2	1.5	38	44

[a] m , σ^2, and SD are mean, variance, and standard deviation respectively, of the distribution function of head capsule widths in each instar.

4.1.2 Minimization of overlap by means of limits.

The discrimination limit is the value that minimizes the sum of the two probabilities of misclassification, as the discrimination point between two contiguous instars (Got, 1988). These discrimination limits were calculated and reported in Table 3. The boundary limit between 1st and 2nd instar was 18.87 µm, between 2nd and 3rd instar was 34.38 µm, and between 3rd and 4th instar was 41.23 µm. These limits were fitted in a graphic of density function (Figure 13), where the largest overlap was found between 3rd and 4th instars.

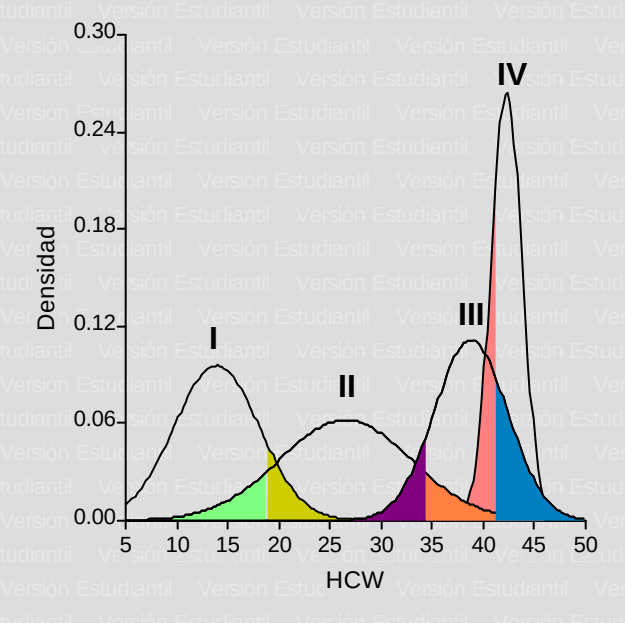

Figure 13. Minimized probabilities of misclassified instars, shown by different colored areas, representing HCW of larvae reared under laboratory conditions.

Table 2 presents the probability of misclassified instars after these limits were applied. The highest probability of misclassification was observed between 4th and 3rd instars (p=0.2476). This means that there exists a 24.76 percent of possibility those cephalic capsules that were considered in the 4th instar, really are in the 3rd. In the same way, the possibility that cephalic capsules that were considered within the 3rd instar may belong to the 4th is 0.2469. The remainder overlapping areas showed a probability of misclassification < 0.12.

Table 2. Probability of misclassification between larval instar of *L. coffeella* under laboratory conditions.

Larval stage	Probability
I – II	0.1170
II – I	0.1168
II – III	0.1093
III – II	0.1096
III – IV	0.2469
IV – III	0.2476

4.1.3 Determination of growth ratios between instars and ranges of HCW for each instar.

In this study, the growth rate was inversely related with each instar (Table 3). This means that HCW of *L. coffeella* did not follows Dyar's (1890) hypothesis of a geometrical growth pattern. The greatest growth ratio was found between the 1st and 2nd instars (1.91). Additionally, days 3, 7 and 11 in the larva's cycle, are the moment in that an instar is changing to the next one (1st to 2nd, 2nd to 3rd, and 3rd to 4th). After that, the larva began the pupal phase.

Table 3. Discrimination limits according to Got (1988) and Catalan (2004), growth ratios between instars according Dyar (1890) and day of the larva's cycle when a determinate instar can be found.

Larval stage	Limit (µm)	Growth ratio Dyar	HCW range (µm)	Day in the larva's cycle
I – II	18.87	1.91	≤18.87	3
II – III	34.38	1.46	18.87 – 34.38	7
III – IV	41.23	1.09	34.38 – 41.23	11

4.2 Determination of larval instars of field collected CLM in Puerto Rico.

4.2.1 Larval instar number

Approximately 3,500 larvae of CLM were analyzed, and the distribution of the total measure of HCW, during January and August (2006 and 2007), is presented in Figure 14. This distribution ranged between 11 µm to 48 µm. Capsules expulsed by the larvae in their last molt were also included. The frequency distribution analysis showed four distinguishable larval instars, with overlap between them. Larvae of 3rd and 4th intars were the most frequently found in the field collected samples, and larvae of 1st instar the lowest one.

Histograms of HCW are also represented individually by year (2006 and 2007) and by season (January and August) (Figure 15). The presence of four peaks in each graph reaffirms the information presented in the histogram with the total data. However, the data from January 2006 (Figure 15a) presents five possible peaks, which are not observed in the others histograms. The instar 5th was not considered in this study because there represented only 0.086% of the observations.

The results of the analysis of variance (Appendix 2), indicates that there are significant differences in the HCW between the studied seasons, but not between the studied years. Larvae of 1st and 4th instars present a mean of HCW significantly higher in August than in January for both years. In general, larvae of instars 1st and 4th were mostly present in January (Figure 14 and Appendix 2), and larvae of 2nd and 3rd instars were mostly present in August. The mean (µm) varied similarly in the successive instars (January and August), and the variance and standard deviation increased throughout the instars for both months. The lower and upper ranges were also very similar for each instar for both cases (Tables 4 and 5).

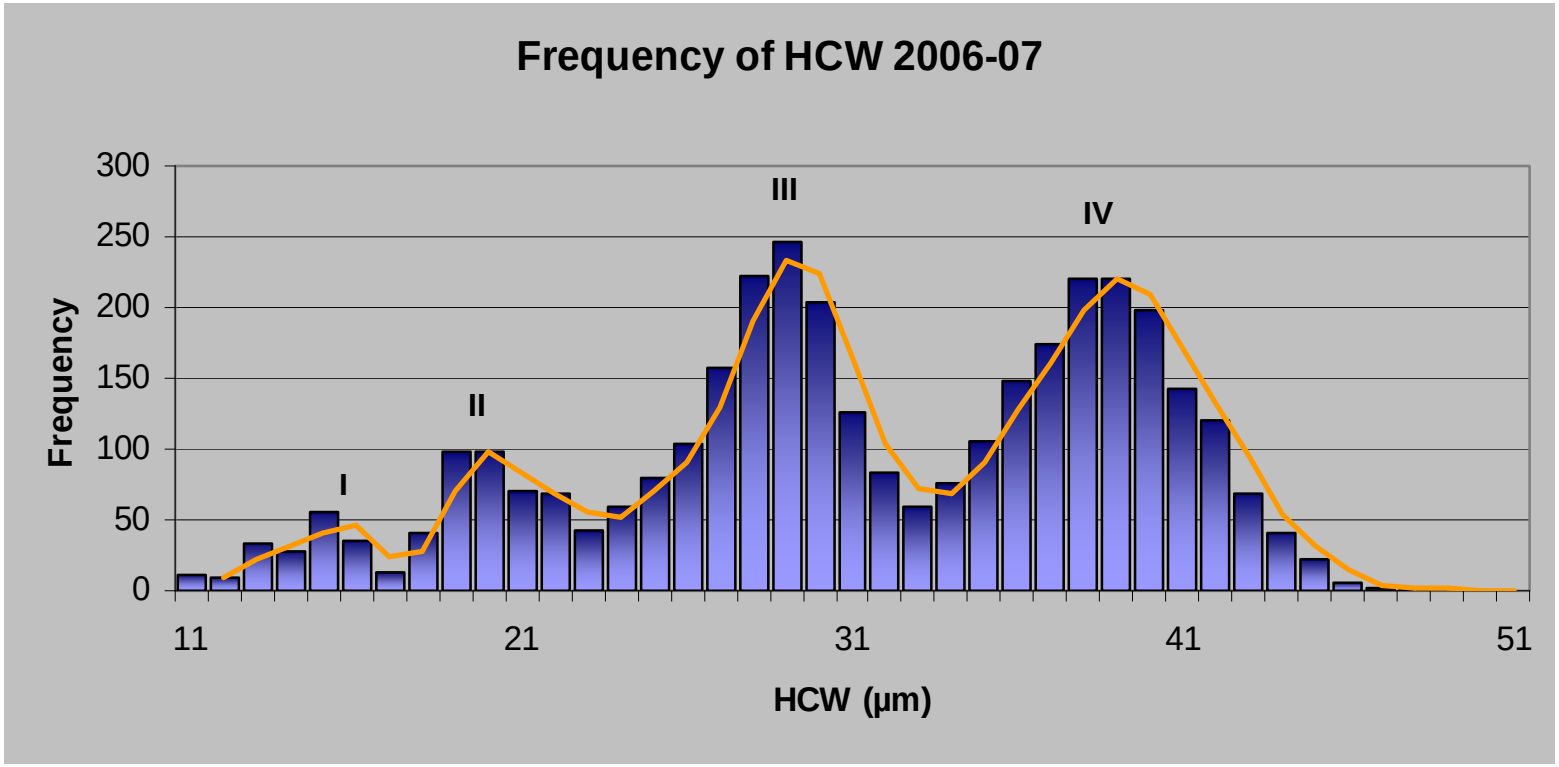

Figure 14 Frequency distribution of head capsule widths of *Leucoptera coffeella* from field samples collected during 2006-07 in Puerto Rico.

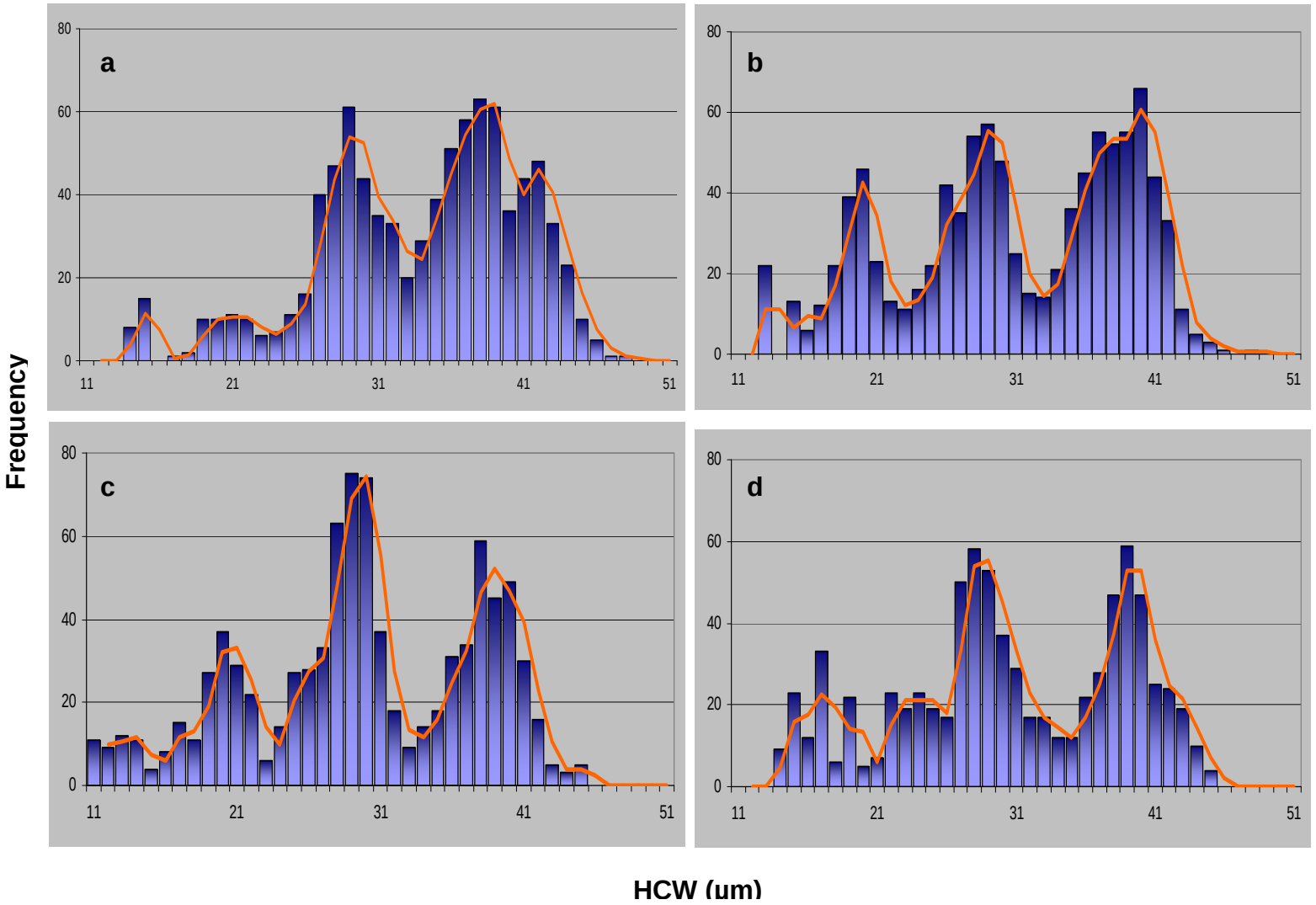

Figure 15. Frequency distribution of head capsule widths of *Leucoptera coffeella* from field collected samples a) January 2006 b) August 2006 c) January 2007 d) August 2007.

Table 4. Descriptive statistics for HCW of *L. coffeella* from field samples collected in January 2006 and 2007 in Puerto Rico.

Instar	n	m[a] (µm)	σ^2 (μm^2)	SD[b] (µm)	Minimun (µm)	Maximun (µm)
I	50	16.8	3.1	1.7	12	18
II	235	21.4	4.4	2.1	19	25
III	711	30.0	7.5	2.7	26	35
IV	807	39.4	6.5	2.5	36	48

[a] m , σ^2, are mean and variance, respectively, of the distribution function of head capsule widths in each instar.
[b] Standard deviation.

Table 5. Descriptive statistic for HCW of *L. coffeella* from field samples collected in August 2006 and 2007 in Puerto Rico.

Instar	n	m[a] (µm)	σ^2 $(\mu m)^2$	SD[b] (µm)	Minimun (µm)	Maximun (µm)
I	145	17.8	1.9	1.4	12	19
II	216	22.2	3.6	1.9	20	25
III	605	29.1	4.8	2.2	26	34
IV	711	38.9	5.7	2.4	35	48

[a] m , σ^2, are mean and variance, respectively, of the distribution function of head capsule widths in each instar.
[b] Standard deviation.

4.2.2 Minimization of overlap by means of limits.

Limits that minimize the possibility of misclassification between instars from samples collected in the field were calculated and are presented in Table 8, and Figures 16 and 17. The highest probability of misclassification was observed between 1st and 2nd instars in January (p=0.1384), and 3rd and 4th instars in August (p=0.2090). The others instars presented a very low probability of misclassification, <0.1 and < 0.17 for January and August samples, respectively.

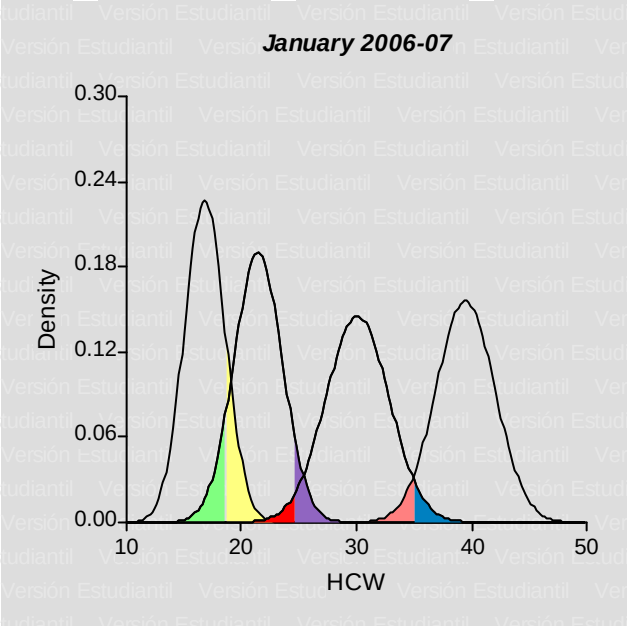

Figure 16. Minimized probabilities of misclassified instars, shown by different colored areas representing HCW of samples collected in January 2006 and 2007, from a field in Puerto Rico.

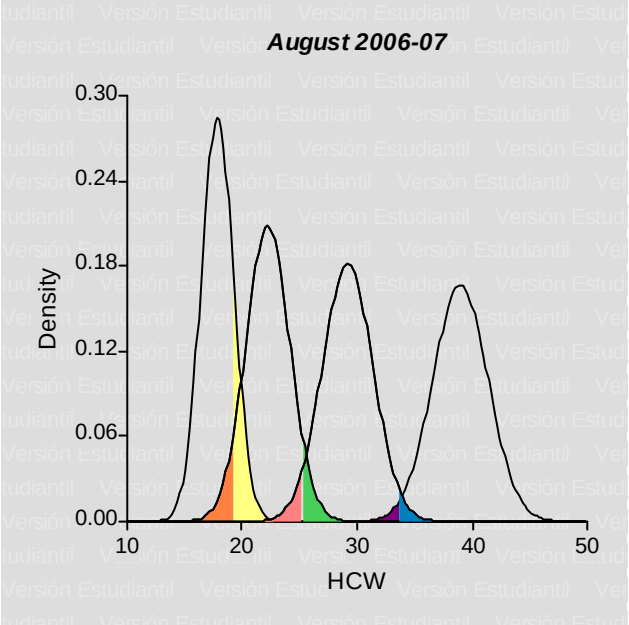

Figure 17. Minimized probabilities of misclassified instars, shown by different colored areas, representing HCW of samples collected in August 2006 and 2007, from a field in Puerto Rico.

Table 6. Probability of misclassification between larval instar of *L. coffeella* from field collected samples in January 2006 and 2007.

Larval stage	Probability
I – II	0.1384
II – I	0.0972
II – III	0.0645
III – II	0.0235
III – IV	0.0338
IV – III	0.0437

Table 7. Probability of misclassification between larval instar of *L. coffeella* from field collected samples in August 2006 and 2007.

Larval stage	Probability
I – II	0.1607
II – I	0.0592
II – III	0.0593
III – II	0.0630
III – IV	0.2090
IV – III	0.0132

4.2.3 Determination of growth ratios between instars, and ranges of HCW for each instar.

Results for samples collected in January (both years) shows a growth ratio of HCW not related to Dyar's rule (Table 8). In contrast, HCW from samples collected in August (both years), presented a pattern related for each instar. For both seasons, the lowest HCW's growth ratio was obtained between 1st and 2nd instars for field collected samples.

Table 8. Discrimination limits in the months of January and August 2006 and 2007 according to Got (1988) and Villa and Catalan (2004), and growth ratios between instars according Dyar (1890).

Larval stage	Limit (µm)		Growth ratio Dyar (1890)	
	January 2006-07	August 2006-07	January 2006-07	August 2006-07
I – II	18.7	19.2	1.28±0.019	1.25±0.021
II – III	24.6	25.2	1.40±0.021	1.31±0.011
III – IV	35.0	33.6	1.31±0.014	1.34±0.072

4.2.4 Comparison of instars distribution of HCW in laboratory-reared and field collected CLM larvae.

The curves presented in Figure 18, compare the *L. coffeella*'s instar distribution between laboratory-reared and field conditions. This figure confirms the information before shows in sections 4.1.1 and 4.2.1, where 4 instars are shown in both conditions. A small difference is observed between ranges of HCW for each instar when both curves are compared. Thus, ranges of HCW, with base in limits (from Got's formula), were higher for 1st, 2nd and 3rd instars, for larvae reared under laboratory conditions, than from larvae collected from field (Table 9). In contrast, larvae of 4th instar presented a higher range of HCW in field conditions than under laboratory-reared. Finally, 1st and 4th instars, and 3rd and 4th instars had the highest HCW under laboratory and field conditions, respectively.

Table 9. Limits of head capsule widths of laboratory reared and field collected coffee leafminer larvae/instar.

Instar	Range (µm)	
	Laboratory conditions	Field conditions
I	≥ 14 - ≤ 18	≥ 11 - ≤ 16
II	≥ 18 - ≤ 28	≥ 16 - ≤ 24
III	≥ 28 - ≤ 38	≥ 24 - ≤ 34
IV	≥ 38 - ≤ 44	≥ 34 - ≤ 48

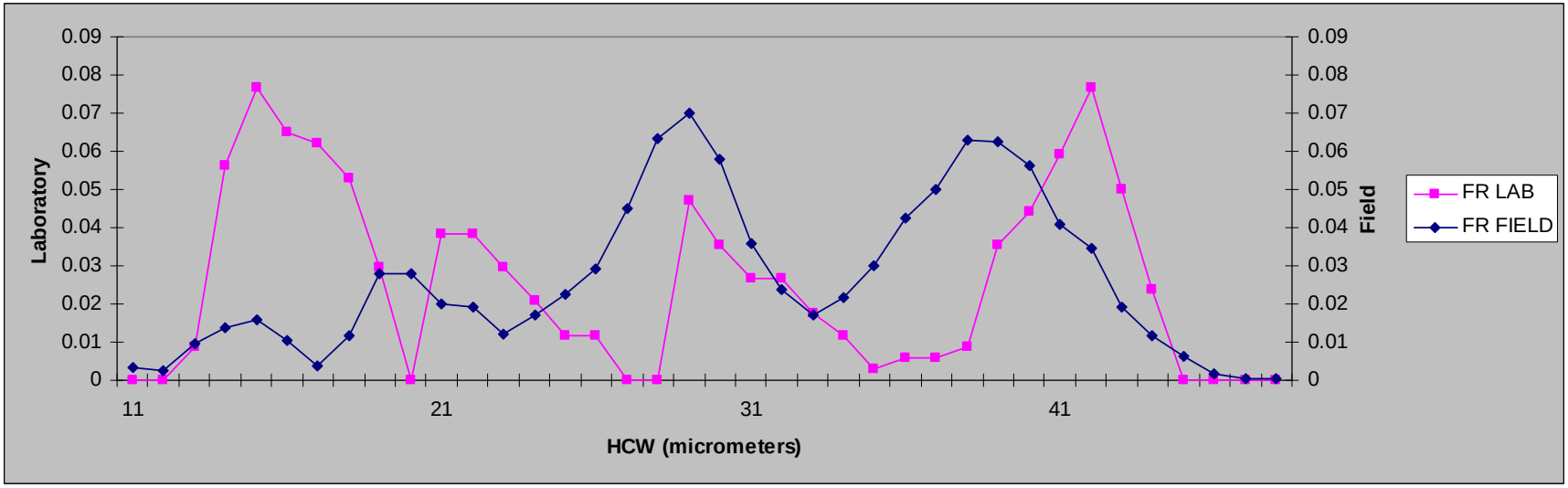

Figure 18. Frequency distribution of measurements of head capsule widths of field-collected and laboratory-reared coffee leafminer in Puerto Rico.

4.2.5 Identification of morphological structures of of *L. coffeella*'s larva.

Some characteristics of the larva were observed by means of an electronic microscope (SEM) (Figures 19 and 20). The larva's body is somewhat flattened in early instars and cylindrical when mature. The thorax has a dark brown pronotum well defined (Figure 19d), with thoracic legs when the larva is mature (Figure 20c). In the third instar a full complement of five pairs of prolegs with crochets is present. The larva's buccal apparatus has a labium, mandible and maxillae all well developed (Figure 19b and 19c). Additionally, identification of each instar of L. coffeella according with the contour line of the head is presented in Figure 21

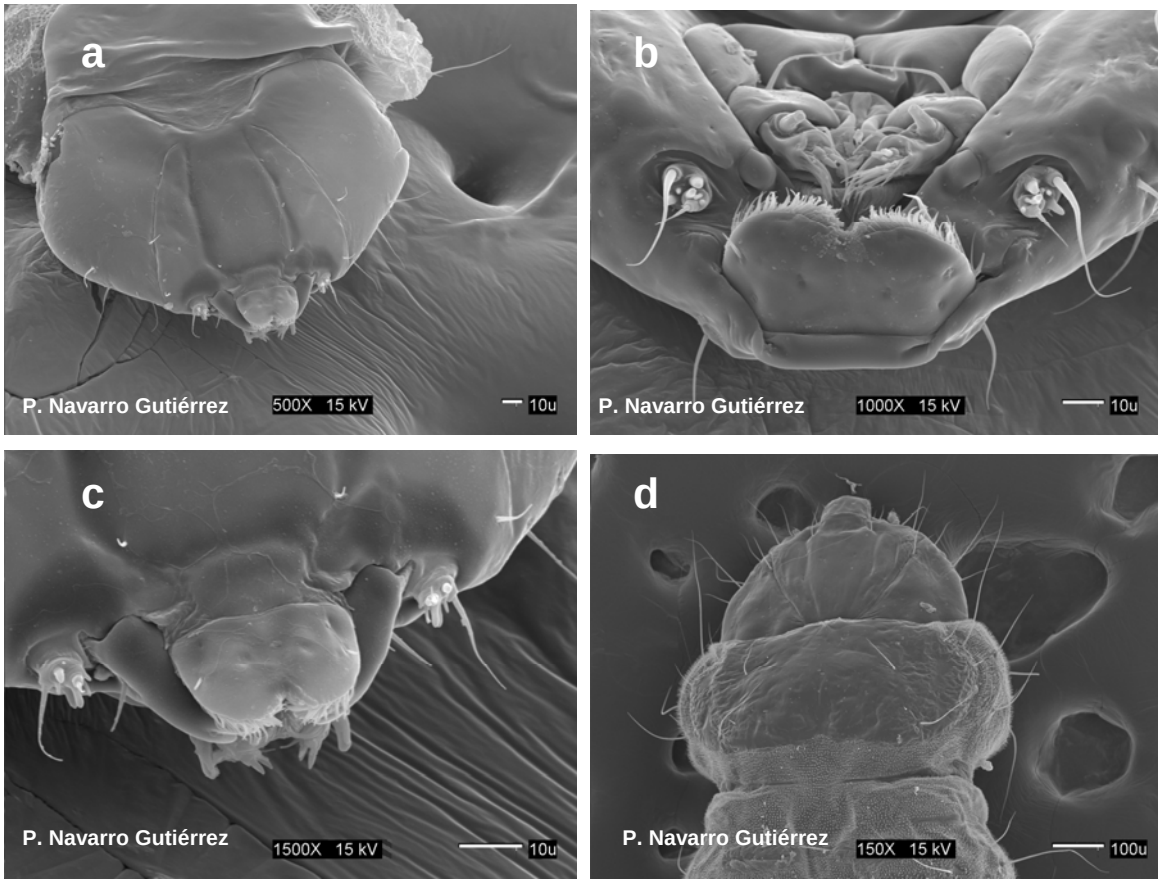

Figure 19. Larvae of *Leucoptera coffeella* observed by electronic microscope (SEM). a) first instar: head, dorsal b) first instar: head, ventral c) third instar: labrum, mandible d) fourth instar: pro-thorax.

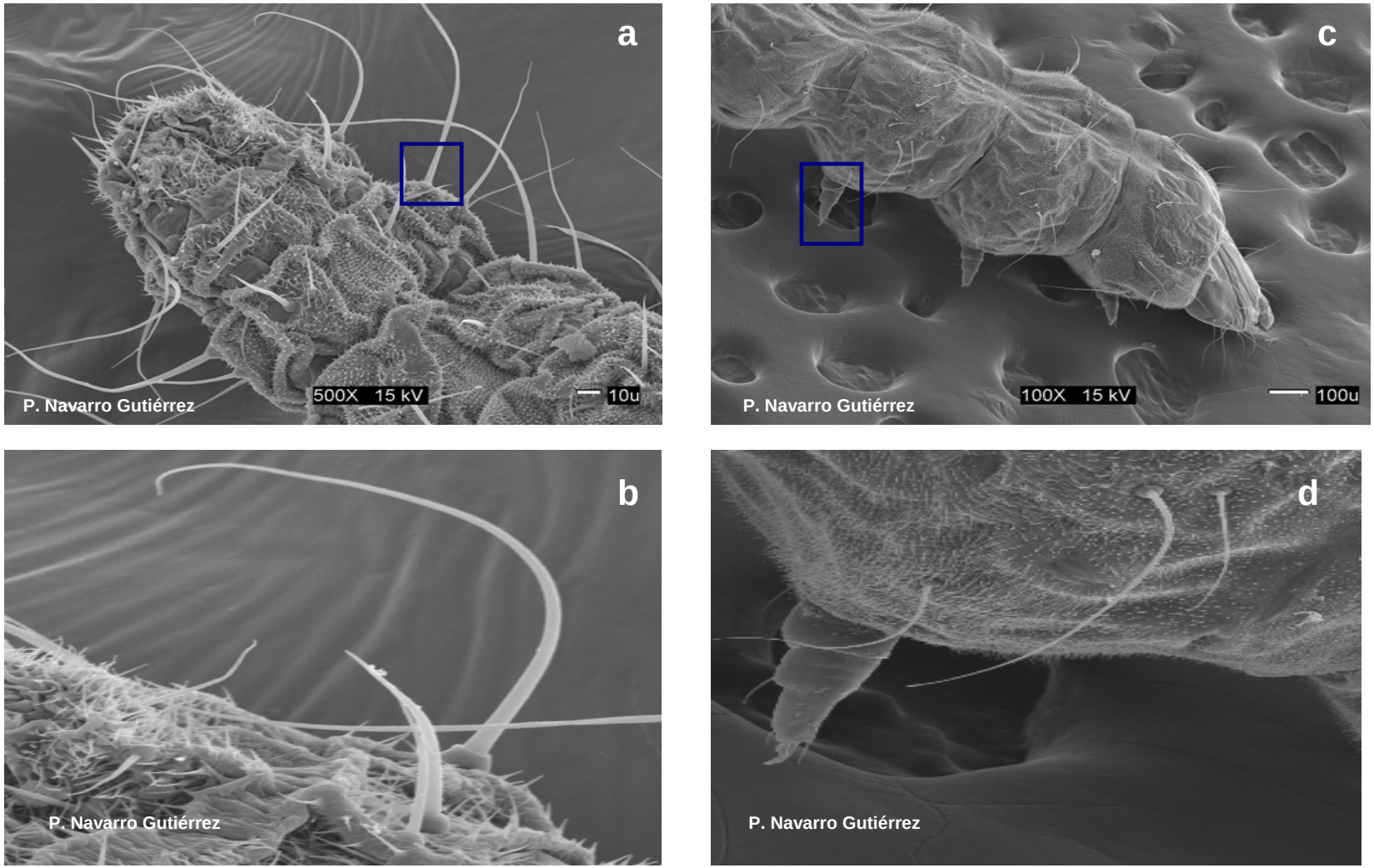

Figure 20. Larvae of *Leucoptera coffeella* observed by electronic microscope (SEM); a) third instar, area inside of the square correspond to stemmatal region and seta which is increased in b), c) third instar, area inside of the square correspond to a proleg which is increased in d).

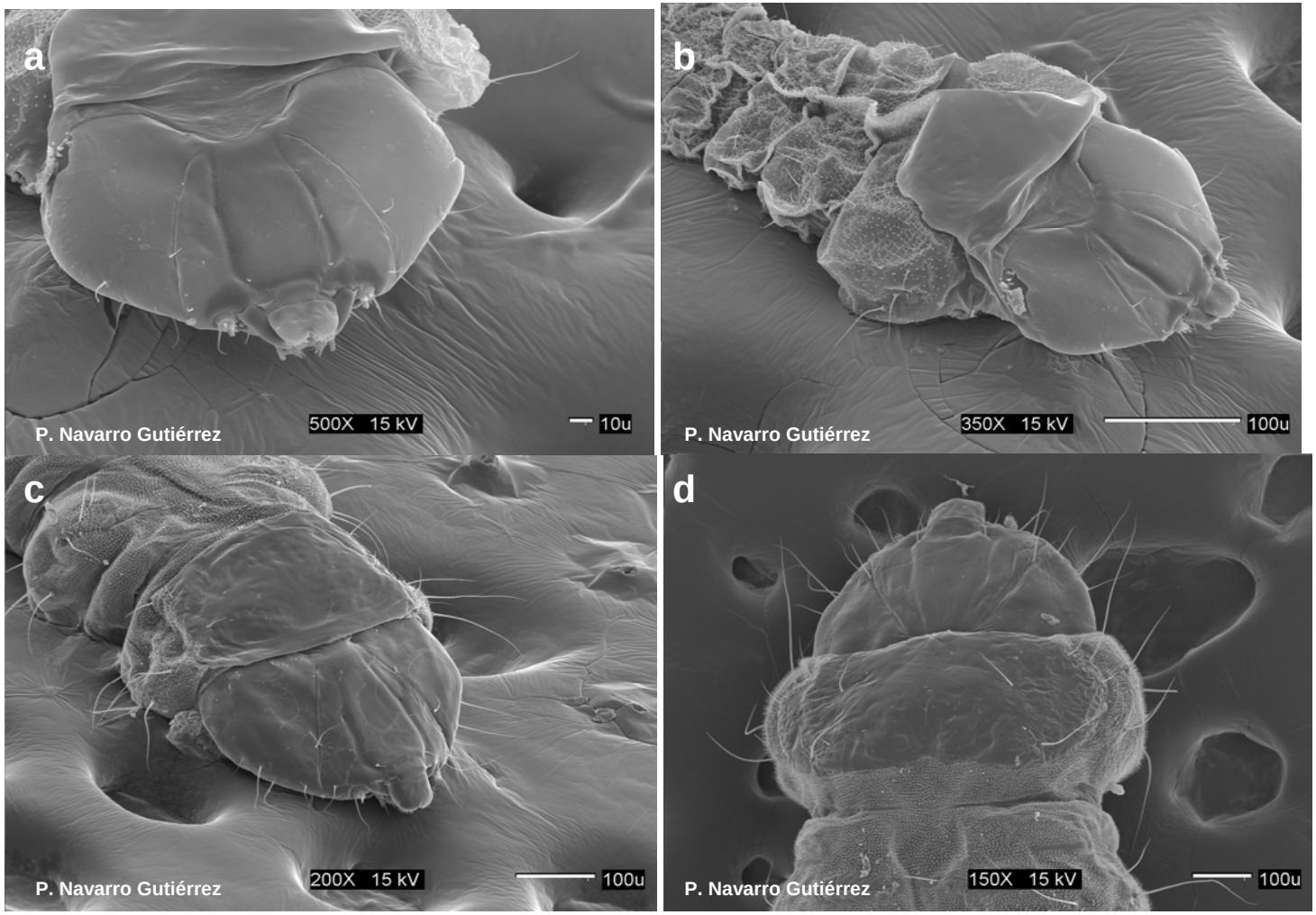

Figure 21. Differentiation of *L. coffeella*'s larval instars according to the angular margin of head a) 1st instar, the contour line of the head is angular; b) 2nd instar, the contour line of the head is less angular than a); c) 3rd instar, the contour line of the head is rounded; d) the contour line of the head is rounder than c).

4.3 Identification of instar (s) of *Leucoptera coffeella* preferred by *Mirax insularis*, for its parasitation, under laboratory conditions.

4.3.1 Relation between the numbers of *Leucoptera coffeella* versus *Mirax insularis* from field collected samples and determination of the sex rates of both species.

The relation between CLM and parasitoids emerged from the field sample and their sex rates are shown in Table 10. To determine the sex ratios in the CLM the last two segments of the abdomen were differentiated (Figure 21 and 22). In the case of *M. insularis* the ovipositor of the female was identified (Figure 23 and 24). *L. coffeella* (63%) was the main emerged species, of which 53% were females; and 52% of *M. insularis* were male.

Table 10. Percent of *Leucoptera coffeella* versus *Mirax insularis* and their sex rates from field collected samples in Puerto Rico.

Species	Percent of the total sample	Female (%)	Male (%)
L. coffeella	63	53	47
M. insularis	25	48	52
Not emerged	12	-	-

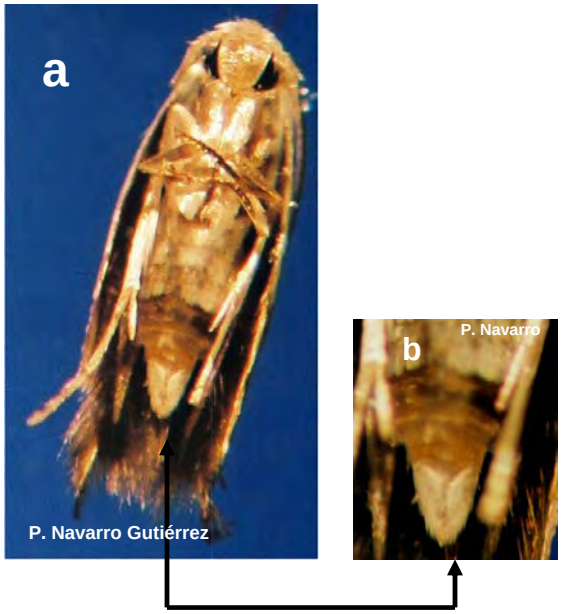

Figure 22. Female of *Leucoptera coffeella*, pictures show a) ventral side b) last two abdominal segments.

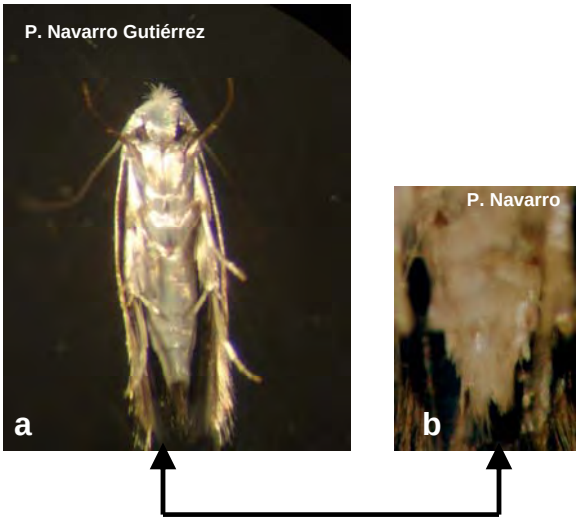

Figure 23. Male of *Leucoptera coffeella*, pictures showing a) ventral side b) last two abdominal segments.

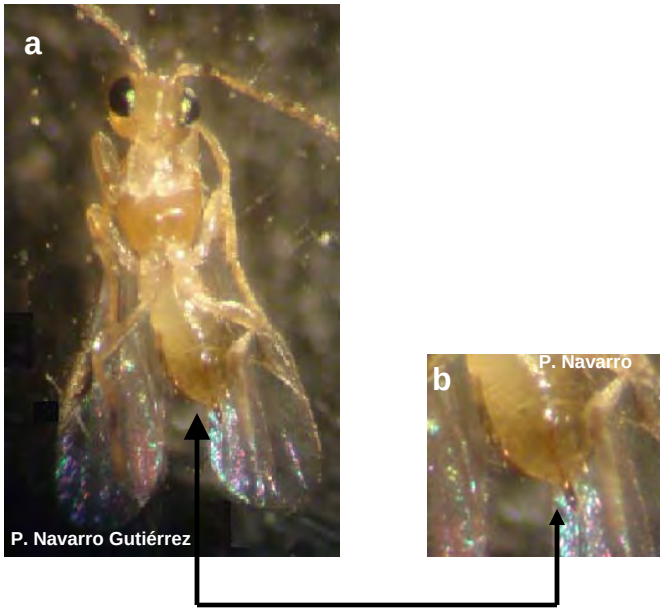

Figure 24. Female of *Mirax insularis*, pictures showing a) ventral side b) ovipositor.

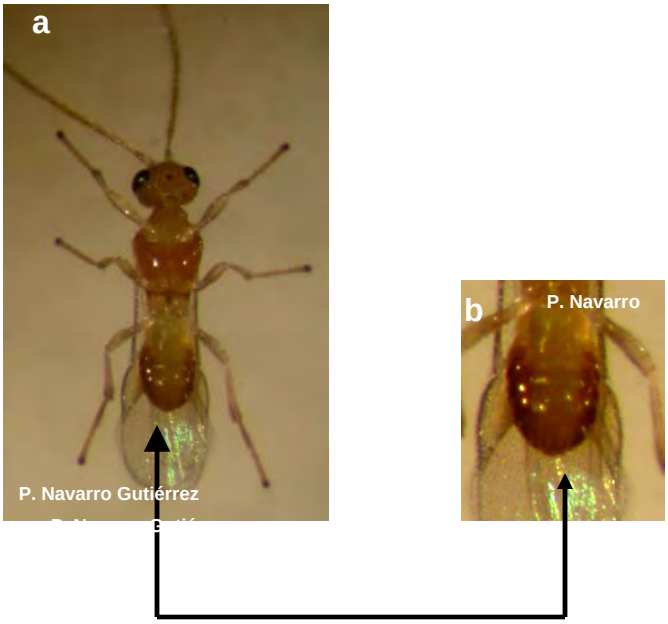

Figure 25. Male of *Mirax insularis*, pictures showing a) ventral side b) last abdominal segment.

4.3.2 Determination of preferred instar (s).

First and second instars of *L. coffeella* had yield the highest percentage of parasitism (60% and 63%, respectively). No parasitism occurred in the 3rd and 4th instars (Table 11). This resulted in a significantly higher selection coefficient of *M. insularis* to the 1st (0.47575) and 2nd (0.51750) instars. No significant differences were observed in the selection coefficient between 1st and 2nd instars. Additionally, different sizes of mines, where each instar can be expected, are presented in Figure 25.

Table 11. Percentage of parasitism and selection coefficient of *Leucoptera coffeella* host parasitized by *Mirax insularis*.

Instar	Percentage parasitism (%)	Selection coefficient
1st	60.00 ± 1.18 a	0.47575 a
2nd	63.00 ± 1.13 a	0.51750 a
3rd	0.07± 0.14 b	0.00000 b
4th	0.00 b	0.00000 b

Note: Means followed by same letter in columns (Tukey's Test) do not differ statistically (α=0.05).

Figure 26. Sizes of mines on the leaves where different instars of *Leucoptera coffeella* can be find it. a) first instar b) second instar c) third instar d) fourth instar.

5. DISCUSSION

5.1 Determination of larval instars of the CLM under laboratory conditions.

There is limited information about the larval instars of *L. coffeella*. Most authors have worked on the effects of temperature on larval development; however, none of these studies have determined CLM instars or their duration. Results of this work shows that *L. coffeella* has four larval instars, under field and laboratory conditions. This result coincides with Notley (1948, 1956), that reports four instars for *L. coffeella*. Another species of this genus that was reported with four larval instars was *L. meyricki* (Bigger and Tapley, 1969). However, *Leucoptera spartifoliella* (Hubner), commonly know as "twig mining moth" had six instars (Herbinson and Crossley, 2004).

There are several factors that must be considered in the CLM's instars determinations. Food availability, temperature and rearing conditions had been reported by Konnorova and Nodarse (1982), and Daly (1985) as factors that can modify the number or duration of the larva's cycle. Food availability may affect growth rates and morphometrics, either between populations or between individuals of the same populations. Additionally, if food and temperature conditions were not optimal, larvae could have shortened their cycle (Daly, 1985). In this study, the duration for each instar was determined using a smooth locally weighted regression. The duration of the larva's cycle was 13 days, which agree with previous reports by Nantes and Parra (1977) and by Konnorova and Nodarse (1982) for CLM. A very similar methodology was used by Hutchinson *et al*. (1997), as a method to predict the effect of environmental manipulation when growth is discontinuous.

The four instars observed under laboratory conditions were overlapping with its adjacent instar. Drozz (1965), Hoxie and Wellso (1974), and Godin et al. (2002) found that the sexual differentiation could be a reason that explain the overlapping between instars of several species. These authors explained that there can be a sexual differentiation beginning at the third or fourth instars, when larger females would create a second peak, immediately after the smaller males. In fact, in this study, the largest overlap was observed between 3rd and 4th instars. Finally, after the limit applications, the probability of misclassification between 3rd and 4th instars was of p=0.247, and to the rest of instars the probabilities were < 0.11.

Results obtained in this study indicate that days 3, 7, and 11 in the larva's cycle are the moment in that an instar changes to the next one. Additionally, it was observed that 2nd and 3rd instars had a longer duration than others, with duration of four days for each one. Variation in the instar duration could be explained because early instars (first and second) of some genera in the Lyonetiidae's family are apodal (Stehr, 1987). When larvae hatch from the egg and begin mining, the absence of legs and prolegs produce a lower mobility, diminishing the possibility to feed itself. Feeding availability of the second instars is lower than the other mature larval stages. For that reason, early instars could require a longer period of time (days) to complete its development and growth, and , start the following instar. In fact, the presence of legs and prolegs in *L. coffeella* was not observed in the early instars of larvae analyzed. The results of this research indicates that the third instar was also longer (days) than the other larval stages. However, the third instar is considered a mature larval stage in this species (Stehr, 1987). This contradicts the previous explanation. Effects of overlaping could have affected these results, considering that some larvae in the third instar may belong to the second.

Dyar's rule states "that the widths of the head (capsule) of a larva in its successive stages follows a regular geometrical progression. If, examining the measurements of heads taken in following out a life, any deviation from the calculated progression, it is evidence that an error has been committed or that the larva has behaved in a abnormal manner" (Camp and Neal, 1993). In the results of this study the growth rates were

decreasing through the instars, denying the influence of time on the growth ratios. Dyar's rule is strongly debated in lepidopteran head capsule analysis. Several authors use this methodology to confirm or reject this hypothesis, according to their own results. On the other hand, Beck (1983) states, that a constant geometric relation in the strict sense is not a feature of the insect's development. In general, growth rates can deviate from linearity, when temperatures approach maximum and minimum tolerable ranges. Others examples where the Dyar's ratio decreases in successive instars were reported by Hoxie and Wellso (1974), Godin *et al*., (2002) and Pantoja *et al*., (2006).

5.2 Determination of larval stage (instars) of field collected CLM in Puerto Rico.

The frequency distribution of HCW is one of the most commonly used analyses to determine the larval instars of lepidopteran species (Caltagirone *et al*., 1983). In this study four distinguishable instars were observed in both cases:1) the histogram that present all the collected data, and 2) histograms with data separated by month and years. An exception was presented in the frequency distribution of January 2006 (Figure 15a), where a possibility of a fifth peak (fifth instar) was observed. This fifth instar could possibly be considered a bimodal peak, produced by different reasons such as sex differentiation (Godin *et al*., 2002), an error in the operator's ability to align the ocular micrometer (Daly, 1985), or that an instar was harder to collect than others (Godin *et al*., 2002). In fact, a low quantity of larvae (n=6), with measures > 44 µm, was considered within of the possible fifth instar. In contrast, like in the others histograms, most of measures of HCW (between 33 and 44 µm approximately) were considered in the fourth instar. Thus, the possibility of a fifth instar was discarded in this study, considering four instars for *L. coffeella* from field collected samples. A similar situation was reported by Godin *et al*. (2002) with the cranberry fruitworm species. Others species of leafminers that reportedly present four instars under field conditions are the potato moth (*Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) (Gilboa and Podoler, 1994), and the citrus leafminer (*Phyllocnistris citrella* (Stainton) (Lepidoptera: Gracillariidae) (Heppner, 1998).

There are several possible explanations for the observed overlap between adjacent instars in this study. Sex differentiation can be a cause. However, no obvious physical differences were observed between sexes in this work, although, they may have been present. According to McClellan and Logan (1994) other factors such as size of sample or sampling method could be influenced. The overlap was considerably diminished by means of boundary limits, in this study. The probability of misclassification instars after of limits application was low. Additionally, the possibility of instar identification is proposed in this study, which can be made by the identification of the contour lines on the larva's head. The contour lines for each instar is presented in Figure 21, where the1st and 2nd instars contour lines are more angular, and the 3rd and 4th instars, contour lines are more rounded.

The HCW's sizes were found to be bigger in August than January, which could indicate better food availability for the larva in this season. However, August is considered within the rainy season in Puerto Rico (Gonzalez, 1996). According to Beck (1972), larvae that survive to the rain season present a harder condition of alimentation. These larvae are able to increase the number of larval stages, and decreasing in size due to food deprivation (Beck, 1972). Both points of view do not coincide in the explanation of biggest sizes of HCW in August. So, other causes such as inexactitude in the measure or, maybe, that larva's food (leaf) is not affected for the rain, must be considered.

Significant differences in the predominant instar were also observed between the studied months. Larvae of 1st and 4th instars, and 2nd and 3rd instars were mostly present in January and August, respectively. Rain and humidity are the most important factors determining the abundance or scarcity of CLM's larva (Notley, 1948). Heavy rain affects the mine when it is split, or has an exit hole, because inside of the mine water fills and the larva in it is drowned (Notley, 1948). This may explain the low proportion of larvae of instar 4 present in August. Additionally, when leaves are wet or with a higher humidity on the surface the CLM'S moths do not lay their eggs on the leaves (Seín, 1940). Thus, the absence of eggs decreases the presence of larvae of first instar. Both

explanations are related with the rainy season, which could be the reason of the population behavior in the field.

Dyar's rule (Dyar, 1890) assumes a geometric progression between mean widths in successive instars. However, in the data collected in January (2006 and 2007) the growth ratio did not follow Dyar's rule. Other studies where the growth ratio of the studied species, did not follow Dyar's rule were reported by Forbush and Fernald (1896), Jobin *et al*. (1992), and McClellan and Logan (1994). In contrast, the results obtained for data collected in August (2006 and 2007), agree with this hypothesis (Table 8). The differences in the growth ratio between January and August could be related to the presence of some kind of stress that affects the normal behavior of the insect's population (Jobin *et al*., 1992). Factors such as availability and quality of food, presence of natural enemies or climatic conditions could be affecting. In fact, in January the leaf's quality is not the best for the larva, because the plant suffers an important stress after harvest. Additionally, in January the humidity in the air is lower than August, which means that the host's population is high and; therefore, the parasitoid's population is also abundant. Thus, according to Jobin et al. (1992), the increased presence of parasitoids can produce stress, affecting the normal behavior of the larvae, by changing its growth rate at body and cephalic capsule.

5.3 Comparison of the distribution of HCW of laboratory-reared and field collected CLM larvae.

Although both populations (laboratory reared and field collected) show four instars, the ranges of HCW for each instar were different. According to Godin *et al*. (2002), factors such as temperature, alimentation, and humidity are directly related with the insect's normal development. when one of these factors has been sub-optimal during the insect's rearing (under laboratory conditions), an abnormal development of the larvae is produced. Under this premise, it was expected that larvae collected from the field had a head capsule (HC) wider than laboratory reared larvae. However, this did not happen because HC of 1^{st}, 2^{nd} and 3^{rd} instars were wider in laboratory reared larvae. In contrast, 4^{th} instar presented a wider HC in larvae from the field. Food and Laboratory conditions were optimal (better than field conditions).

Finally, 1^{st} and 4^{th} instars, and 3^{rd} and 4^{th} instars were mostly present under laboratory and field conditions, respectively. This information has not been obtained before for *L. coffeella*, but agrees with results obtained by Godin et al., (2002) for field collected samples of the cranberry fruitworm (Lepidoptera:Piralidae).

5.4 Identification of morphological structures of the larva of *L. coffeella*.

L. coffeella's larvae have a relatively depressed head and presents a cylindrical body in mature instars (Figures 19a and 20c, respectively) which coincide with the characterization given by Stehr (1987). The author mentioned that these characteristics are typical in the Lyonetiidae larvae, and that the early leaf-mining instars of many, if not all, genera are apodal. The apodal condition was present in the larvae of the analyzed samples and is presented in the Figure 26 and 27. This study also confirmed the presence of prolegs in the third instar (Figure 20c). From this stage on, the larva continues mining and enlarging the mine to form large blotches (Stehr, 1987). Mature larvae measured between 5 to 10 millimeters presenting a somewhat flattened body in early instars and cylindrical when matured. The body's color ranged from translucent to white with a pronotum well defined and dark brown (Figure 19d).

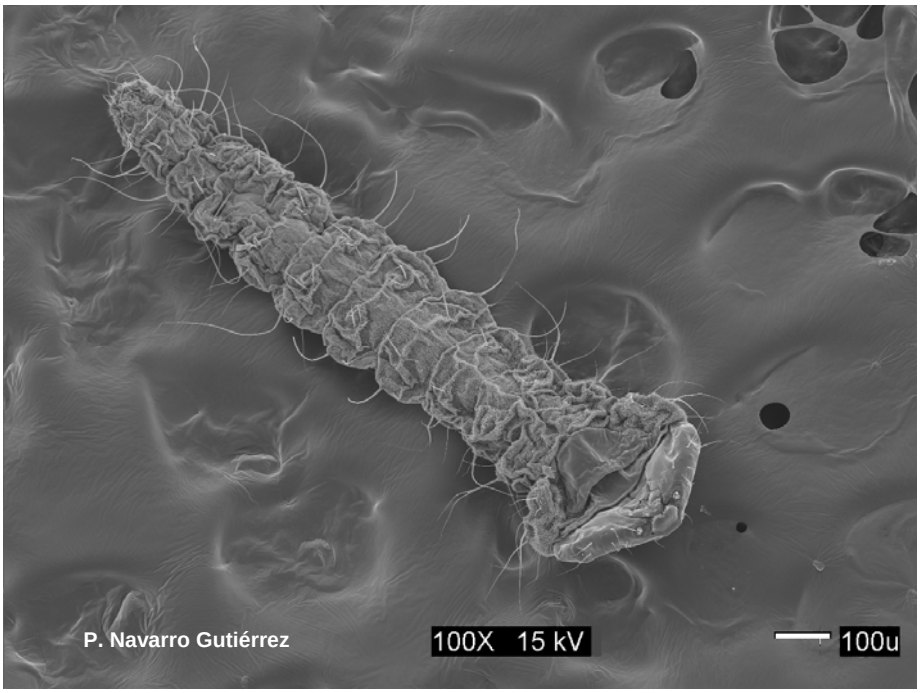

Figure 27. *Leucoptera coffeella* ventral side, a) shown the apodal condition of the second instar.

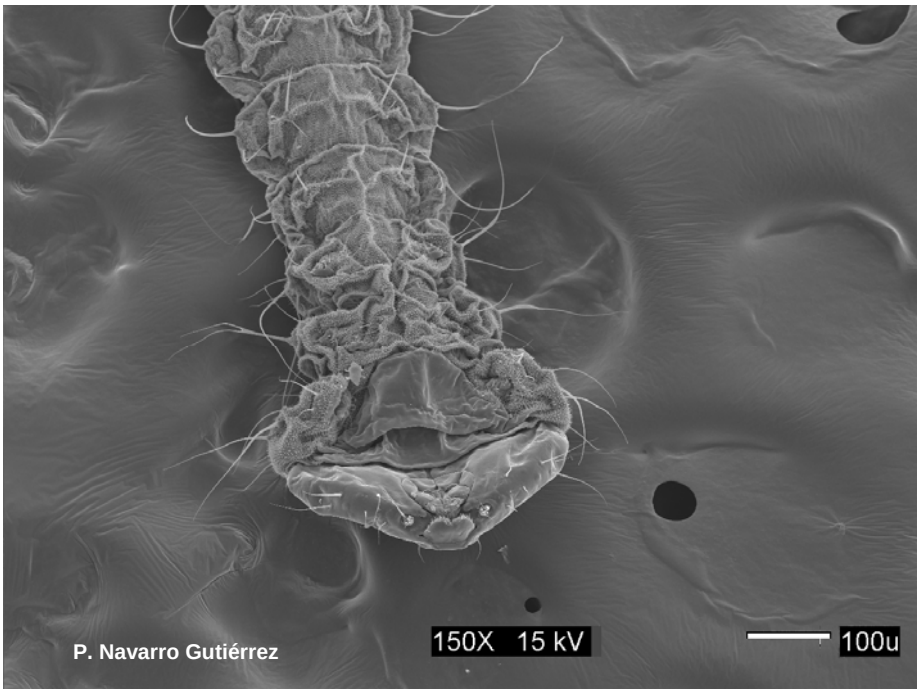

Figure 28. *Leucoptera coffeella* ventral side, shown absence of legs in the second instar.

5.5 Identification of instar (s) of *L. coffeella* preferred by *M. insularis*, for its parasitization under laboratory conditions.

In this study, under laboratory conditions, 1st and 2nd instars of *L. coffeella* were parasitized by *M. insularis* in 60% and 63%, respectively. This was corroborated with the highest selection coefficient obtained to these larval stages. In contrast, in 3rd and 4th instars, parasitization was not observed. According to Li *et al*. (2006), the nutrition quality may be different in the different host instars. Pennacio *et al*. (1992) states that there are differences in the host's quality with increasing age, which affect the developmental performance for the larval parasitoid.

M. insularis's cycle has a duration between fifteen and seventeen days (Leon, 1997), which is very similar to the host larval cycle. *M. insularis* is a koinobiont endoparasitoid that develops on its host and continues to feed and grow after parasitization (Kuriachan *et al*., 2006). When parasitization has occurred the parasitoid requires that the larva remain alive throughout the parasitoid's development.

Microplitis mediator (Hymenoptera: Braconidae) is another Braconidae that has been reported parasitizing the CLM's second instars. Li *et al*. (2006), reported that no *M. mediator* was found in the 5th instar. This supports the concept that the host's immune system is strong enough by the 5th instar to prevent the development of the parasitoid. This may be applied to the results obtained in this study, for 3rd and 4th instars of *L. coffeella*. These instars could be strong enough to prevent the parasitization. In contrast, 1st and 2nd instars were more susceptible to be parasitized, and were preferred by *M. insularis.* Instars 1 and 2 provide a better nutritional quality to the parasitoid than the others instars.

Based on the results obtained in this study, 1st and 2nd instars are recommended to mass rear *M. insularis*, to optimize the percentage of parasitism, parasitoid development and survival. Additionally, when augmented field releases of *M. insularis* are conducted, the parasitoids should be released during the first and second instars of *L. coffeella*, which according to this study are mainly present in August (Figure 15a, b c and d). In larvae collected in field conditions instars 3rd and 4th showed the highest probability of overlap. However, this is not a problem in the synchronization process because these instars are not parasitized by *M. insularis*.

6 CONCLUSIONS

1. *Leucoptera coffeella* presented four larval instars under laboratory conditions with a total duration of 13 days.

2. Instars 1 and 4 were the most common present under laboratory-reared conditions.

3. The period of each instar under laboratory condition were: first instar between days 1 and 3, second instar between days 3 and 7, third instar between days 7 and 11, and fourth instar between days 11 and 13.

4. Limits of HCW between instars for *L. coffeella* under laboratory conditions are: 18.8 µm between the 1st and 2nd instars, 34.3 µm between the 2nd and 3rd instars, and 41.2 µm between the 3rd and 4th instars.

5. The greatest possibility of misclassification, under laboratory conditions, is between instars 3 and 4, in both directions, with 24% of probabilities for each.

6. Dyar's growth ratio is inversely related to the HCW of larvae reared under laboratory conditions.

7. Days 3, 7 and 11, in the larva's cycle, are the moments when one instar changes to the next one.

8. *Leucoptera coffeella* presented four larval instars in the field in Puerto Rico.

9. Larvae of 1st and 4th instars were mostly collected in January, and larvae of 2nd and 3rd were mostly present in August.

10. For samples collected in the field, the highest probability of misclassification was observed between 1st and 2nd instars in January and 3rd and 4th instars in August.

11. The observed overlap between 3rd and 4th instars (in larvae collected at the field), do not affect in the synchronization process, because 3rd and 4th instars are not parasitized by *M. insularis.*

12. Dyar's growth ratio was followed in samples collected in August (2006 and 2007), but not in samples collected in January of the same years.

13. First and second instars of *L. coffeella* were preferred by *M. insularis* for its parasitation, with a 60% and 63% percent of parasitation, respectively.

14. The selection coefficient for instars 1 and 2 were 0.48 and 0.52 respectively.

15. Instars 3 and 4 of *L. coffeella* do not present parasitism by *M. insularis*.

16. Finally, with the information obtained from this study, it will be possible to synchronize the parasitoid *Mirax insularis* with the coffee leafminer *L. coffeella* at the strategic moment. This permit the implementation of a augmentation program of this parasitoid, which gives the possibility to reduce the use of chemicals for the control of this pest. Environmental and economic benefits are expected in Puerto Rico and other countries where coffee is cultivated.

7 RECOMMENDATIONS

1. Study if sex differentiation in larvae is present or not.

2. Study the possibility that different biotypes of *L. coffeella* could be present in the coffee grown in Puerto Rico.

3. Use 1st and 2nd instars of *L. coffeella* to mass rear *M. insularis* and optimize the percentage of parasitism, parasitoid development, and survive.

4. Parasitoids should be released in August when the first and second instars of *L. coffeella* are highly present on the field.

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APPENDICES

APPENDIX 1. Descriptive statistics for the development of head capsule widths in the specie *L. coffeella* during its life larval cycle under laboratory conditions.

Day	n	Mean (±SD) (µm)	Lower (µm)	Upper (µm)	Rate of growth) (Dyar 1890
1*	20	10.95 (± 6.55)	0	16	---
2	20	15.00 (± 0.65)	14	16	1.37
3	20	15.30 (± 2.45)	14	25	1.02
4	20	21.50 (± 5.68)	14	34	1.41
5	20	24.95 (± 3.94)	19	32	1.16
6	20	28.55 (± 5.95)	21	41	1.14
7	20	30.25 (± 6.36)	15	41	1.06
8	20	35.60 (± 4.60)	29	42	1.18
9	20	40.05 (± 2.65)	31	44	1.13
10	20	40.85 (± 1.50)	36	42	1.02
11	20	42.15 (± 1.31)	38	44	1.03
12	20	42.15 (± 1.69)	39	44	1.00
13	20	42.60 (± 1.45)	40	44	1.01

***48 hours after oviposition.

APPENDIX 2 Means of HCW of the specie *L. coffeella* by instar to the months of January and August of 2006-07.

Season	Year	Instar	Mean	n	
January	2007	1	12.53	43	A*
January	2006	1	13.00	3	A
August	2007	1	14.00	9	AB
August	2006	1	14.80	10	B
August	2007	2	18.27	101	C
August	2006	2	19.00	119	CD
January	2007	2	19.11	131	CD
January	2006	2	20.32	44	D
January	2007	3	24.58	259	E
August	2006	3	26.22	273	EF
August	2007	3	26.84	238	FG
January	2006	3	27.94	232	G
January	2007	4	35.11	522	H
August	2006	4	37.09	530	I
August	2007	4	37.12	416	I
January	2006	4	37.97	592	I

* Data were analyzed with ANOVA and Tukey's multiple range test.
* Values within a column followed by the same letter are not significantly different (P>0.05).