Population Monitoring and Morphometrics of *Helicoverpa armigera* (Hübner), *Helicoverpa zea* (Boddie), and Their Hybrids (Lepidoptera: Noctuidae) in Puerto Rico

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ABSTRACT

Helicoverpa armigera and *H. zea* are serious pests in many crops; *H. armigera* was detected in 2014 in Puerto Rico. In the period February 2016 to January 2017, 112 traps with a lure for *H. armigera* were installed in Guanica, Santa Isabel, Guayama, Isabela, Lajas, Juana Diaz, Añasco, Aguadilla, Sabana Grande, Mayagüez, San Juan, Gurabo, Villalba, and Jayuya. The two lepidopterans were identified with morphology. Insects identified as *H. armigera* with morphology were corroborated with molecular tools. Comparisons of *Helicoverpa* populations between localities and among crops in each locality were done with non-parametric tests. Data of moth capture by a bell pepper farm in Santa Isabel was used to analyze relationship with weather variables. A total of 5,122 *Helicoverpa* specimens were captured, four were identified as *H. armigera*, and the remaining were *H. zea*. In San Juan, Gurabo, Villalba, and Jayuya any *Helicoverpa* was captured. The highest *H. zea* populations were in the period February to May. Santa Isabel had the highest *H. zea* populations. In the comparisons among crops, only okra in Guanica had higher *H. zea* populations over sunflower. In Santa Isabel, populations of *H. zea* had a significantly negative relationship with maximum temperature, and precipitation depending on the period analyzed. F1 hybrids between *H. armigera* and *H. zea* were obtained at the Center for Excellence in Quarantine and Invasive Species. For morphological comparison, the following variables were analyzed: number of lobes, number of cornuti, length of valves, length of aedeagus, and depth in the posterior excavation in the eighth sternite. The species *H. armigera* is characterized by the presence of one lobe while F1 hybrids and *H. zea* showed three lobes. Statistical analysis with the other quantitative variables showed that *H. zea* had the biggest structures or more cornuti, followed by the hybrids, and finally *H. armigera* with the smallest size of structures and a minor number of cornuti. The principal components analysis of quantitative variables showed that F1 hybrids were closer to *H. zea*. Currently, we can infer that using only morphology is very difficult to identify one *Helicoverpa* as a hybrid or distinguish a true hybrid from a parental species.

RESUMEN

Helicoverpa armigera y *H. zea* son plagas importantes en muchos cultivos; *H. armigera* fue detectada en el 2014 en Puerto Rico. En el periodo de febrero 2016 a enero 2017, se instalaron 112 trampas con feromona para *H. armigera* en Guánica, Santa Isabel, Guayama, Isabela, Lajas, Juana Díaz, Añasco, Aguadilla, Sabana Grande, Mayagüez, San Juan, Gurabo, Villalba, y Jayuya. Ambos lepidópteros se identificaron con morfología. Los insectos identificados como *H. armigera* con morfología se corroboro su identificación con marcadores moleculares. Las comparaciones de las poblaciones de *Helicoverpa* entre localidades y entre cultivos en cada localidad se realizaron con pruebas no paramétricas. Los conteos de *Helicoverpa* en una finca de pimiento en Santa Isabel se usaron para analizar la relación con variables climáticas. Se colectó un total de 5,122 *Helicoverpa,* cuatro se identificaron como *H. armigera,* el resto de los insectos se identificaron como *H. zea.* En San Juan, Gurabo, Villalba, y Jayuya no se capturó ninguna *Helicoverpa.* Las poblaciones más altas de *H. zea* fueron en el periodo de febrero a mayo. En Santa Isabel se registraron las poblaciones más altas de *H. zea.* En las comparaciones entre cultivos, el cultivo de okra registró mayores poblaciones que girasol en Guánica. En Santa Isabel, las poblaciones de *H. zea* tuvieron una relación significativa negativa con la temperatura máxima, y con la precipitación dependiendo del periodo analizado. Híbridos F1 entre *H. armigera* y *H. zea* se obtuvieron en el Centro de Excelencia en Especies Cuarentenarias e Invasivas. Para la comparación morfológica se analizaron las siguientes variables: número de lóbulos, número de cornuti, longitud de valvas, longitud del aedeago, y la profundidad de la excavación posterior del octavo esternito. *Helicoverpa armigera* se caracterizó por la presencia de un lóbulo, mientras en los híbridos F1 y *H. zea* se observaron tres lóbulos. En los análisis estadísticos con el resto de las variables cuantitativas, *H. zea* tuvo las estructuras más grandes o más cornuti, seguido de los híbridos, y finalmente *H. armigera* con las estructuras más pequeñas o menor número de cornuti. El análisis de componentes principales de las variables cuantitativas mostró que los híbridos tuvieron mayor similitud con *H. zea.* Es muy difícil identificar una *Helicoverpa* como híbrido usando solamente la morfología o distinguir un verdadero híbrido de sus especies parentales.

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CHAPTER I. General introduction

1. Introduction

The genus *Helicoverpa* (Lepidoptera: Noctuidae: Heliothinae) consists of 20 species; *H. armigera* (Hübner) and *H. zea* (Boddie) are the most important pests for agriculture. Other economic important species affecting crops are *H. punctigera* (Wallengren), *H. assulta* (Guenée), and *H. gelotopoeon* (Dyar) (Gordon et al, 2009). *Helicoverpa armigera, H. zea, H. punctigera,* and *H. gelotopoeon* are polyphagous species, while *H. assulta* affects only solanaceous plants (Hardwick, 1965). *Helicoverpa armigera* could affect more than 180 plant species. *Helicoverpa zea* attack 123 hosts (Cunningham & Zalucki, 2014; Kogan, et al., 1989). *Helicoverpa assulta* is present in Australia, Asia, and Africa. *Helicoverpa gelotopoeon* is reported in South America. *Helicoverpa punctigera* is an endemic species of Australia (Gordon et al., 2009). *Helicoverpa zea* is widespread in America, including Puerto Rico (Hardwick, 1965). *Helicoverpa armigera* was distributed in the Old World, since 2013 was reported in America, in 2014 was detected in Puerto Rico (Kriticos et al., 2015).

The larvae feed on flowers, fruits, and sometimes on the leaves of its hosts (Hardwick, 1965). The last instar causes more than 80 % of the damage (Nibouche et al., 2007). In the United States, *H. zea* was considered the third most destructive pest (Hardwick, 1965); the damage of *H. zea* with *Chloridea (Heliothis) virescens* (Fabricius) (Lepidoptera: Noctuidae) is estimated over one billion dollars per year in the United States. Annual losses due to *H. armigera* are estimated at 5 billion dollars around the world (Gowda, 2005). In India, the losses on legumes caused by *H. armigera* are estimated at 300 million dollars per year (Fitt, 1989). In India and China, approximately 50 % of pesticides used are to control *H. armigera* (Lammers & Macleod, 2007). In Brazil, in the season 2012/2013 was estimated that *H. armigera* caused damage near to 800 million dollars (Freitas Bueno & Sosa-Gómez, 2014). *Helicoverpa armigera* and *H. zea* have developed resistance to some groups of insecticides, mainly pyrethroids, organochlorines, organophosphates, carbamates, and toxins from the bacterium *Bacillus thuringiensis*. For *H. armigera* there are more reports of resistance than *H. zea*, and resistance factors are higher for *H. armigera* (Kranthi, et al, 2005; Abd Elghafar, et al, 1993; Kanga, et al., 1996; Mahon, et al., 2007; Pérez et al., 2000; Pietrantonio et al., 2007; Ali, et al., 2006). The life cycle of *H. armigera* is shorter than *H. zea* (Barbosa et al., 2016).

For these reasons, the recent reports of the introduction of *H. armigera* in America are a challenge for agriculture.

Helicoverpa armigera and *H. zea* are considered twin species because their external morphology is identical. Identification of these insects must be done with male genitalia or using molecular tools (Perera et al., 2015; Pogue, 2004). Behere et al. (2007) analyzed single nucleotide polymorphisms (SNPs) in the cytochrome oxidase subunit I (COI) region, their data suggest that *H. zea* derived from *H. armigera* around 1.5 million years ago. Pearce et al. (2017) found that in this process of derivation, *H. zea* lost some detoxification genes, and certain genes that confer resistance to insecticides; this explains why *H. armigera* is more polyphagous and more resistant to insecticides than *H. zea. Helicoverpa armigera* shows more levels of genetic diversity than *H. zea* (Leite et al., 2016; Mallet et al., 1993).

With the recent introduction of *H. armigera* in Puerto Rico, it is necessary to research its biology, population dynamics, detection techniques, resistance to insecticides, possible hybridization events with *H. zea*, natural enemies, tactics of management, and other aspects to conduct a good Integrated Pest Management (IPM) for this new pest in the island.

2. Biology of *Helicoverpa armigera* and *H. zea*

Helicoverpa has complete metamorphosis, its life cycle is egg, larva, pupa, and adult. Females lay their eggs mainly in the flowers of their hosts. These eggs hatch in three to seven days. *Helicoverpa* complete the larval stage on five to seven instars (Hardwick, 1965;). Under laboratory rearing conditions, duration time larvae of *H. armigera* (12.7 ± 0.3 days) is statistically less than *H. zea* (17.7 ± 0.6 days) (Barbosa et al., 2016). The mature larvae drop to the soil and burrow into the ground to form their pupal cell. Pupal stages of *Helicoverpa* have a similar duration of 9 to 25 days (Mironidis & Savopoulou-Soultani, 2008). Pupae could enter in a facultative diapause, this is a period of dormancy, during which growth, and metamorphosis cease (Hardwick, 1965; Triplehorn & Johnson, 2005). Diapausing *Helicoverpa* pupae are more tolerant to cold and dry conditions (Fitt, 1989). Adults have nocturnal habits, they usually feed and copulate during hours of darkness (Hardwick, 1965). *Helicoverpa* adults could migrate up 1000 km (Fitt, 1989; Westbrook & López, 2010).

Helicoverpa armigera could attack more than 180 plant species of 68 families. This insect is an important pest in crops such as cotton *(Gossypium hirsutum* L.), tobacco *(Nicotiana tabacum*

L), pigeon pea *(Cajanus cajan* L.), chickpea *(Cicer arietinum* L.), tomato *(Solanum lycopersicum* L.), soybean *(Glycine max* L.*)*, sunflower *(Helianthus annuus* L.), bell pepper *(Capsicum annuum* L.), rice *(Oryza sativa* L.), maize *(Zea mays* L.) and others (Cunningham & Zalucki, 2014).

Helicoverpa zea is reported affecting 123 hosts of 29 families. This species affects mainly corn, sorghum *(Sorghum bicolor* L.), cotton, tomato, sunflower, and soybean (Cunningham & Zalucki, 2014; Fitt, 1989). Hardwick (1965) indicates that *H. zea* prefers corn to any other host. In Puerto Rico, *H. zea* has been reported in: corn, tomato, pigeon pea, bell pepper, sunflower, tobacco, bean *(Phaseolus vulgaris* L.*)*, pea *(Pisum sativum* L.*)*, sugarcane *(Saccharum officinarum* L.), eggplant *(Solanum melongena* L.), and watermelon (*Citrullus lanatus* L). Corn and tomato are the hosts more affected by *H. zea* in Puerto Rico (Calero-Toledo, 2007; Martorell, 1976).

3. *Helicoverpa armigera* in the New World

Helicoverpa armigera, Old World Bollworm (OWB), was distributed in Europe, Africa, Asia, Oceania, and on South-West islands of the Pacific Ocean (Hardwick, 1965). In the last decade, *H. armigera* was found attacking crops in the American continent. The first detection of this species in America was in Brazil in 2013 in a soybean field (Czepak & Albernaz, 2013); but is possible that this pest arrived in Brazil before, Sosa-Gómez et al. (2016) identified larvae of *H. armigera* collected in Brazil in 2008. This pest in Brazil is from multiple origins: Asia, Europe, and Africa (Anderson et al., 2016; Tay et al., 2017). In 2014, *H. armigera* was detected in Argentina in yellow Unitraps with P037-Lure specific for *H. armigera* (ChemTica) placed in chickpea fields (Murúa et al., 2014). Arnemann et al. (2016) confirmed with molecular tools that *H. armigera* is present in Uruguay and Paraguay, Arneman et al. (2016) collected the insects in delta traps with pheromone ISCAlure armigera. Kriticos et al. (2015) indicate that *H. armigera* is present in Bolivia. Gilligan et al. (2015) identified *H. armigera* from the Dominican Republic and Peru. In September 2014, a male adult of *H. armigera* was detected in Puerto Rico (USDA-APHIS-PPQ, 2014). In June 2015, one male *H. armigera* was detected in a pheromone trap in Florida (Hayden & Brambila, 2015).

In Brazil, *H. armigera* is apparently predominant above *H. zea* in dicotyledonous hosts, and *H. zea* is predominant in corn (Leite et al, 2014; Bentivenha et al., 2016). Freitas Bueno & Sosa-Gómez (2014) suggest that *H. armigera* is more aggressive in dry and warm states of Brazil; on the other hand, Leite et al. (2014) did not found a difference in the populations of *H. armigera* in winter

and summer in four states in Brazil. So far, *H. armigera* is reported in 16 of 27 states in Brazil. This pest has been reported in Brazil in the following crops: soybean, Bt-soybean, cotton, Bt-cotton, bean, tomato, corn, millet *(Eleusine coracana* Gaertn), sorghum, citrus, and fly bush *(Plectranthus neochilus* Schltr) (Leite et al., 2014; Bentivenha et al., 2016; Santos, 2015*;* Mastrangelo et al., 2014*;* Pinto et al., 2017*;* Sosa-Gómez et al., 2016*;* Bueno et al., 2014*;* Krinski & Godoy, 2015). Durigan et al. (2017) report failures in the management of *H. armigera* with pyrethroid insecticides deltamethrin and fenvalerate in central and northeastern Brazilian states, populations of *H. armigera* in these states are tolerant to deltamethrin and fenvalerate. Murúa et al. (2016), report that in Argentina *H. gelotopoeon* is apparently predominant above *H. armigera* and *H. zea* in chickpea and soybean*; H. armigera* currently is present in eight of twenty-three provinces of Argentina. *Helicoverpa armigera* has been documented in Argentina attacking the following crops: chickpea, soybean, cotton, corn, sunflower, and spiny plumeless thistle *(Carduus acanthoides* L.) (Arneodo et al., 2015; Murúa et al., 2016). There are reports of *H. armigera* in other American countries: Uruguay, Paraguay, Bolivia, Dominican Republic, Peru, Puerto Rico, and the continental United States (Arnemann et al., 2016; Hayden & Brambila, 2015; Kriticos et al., 2015; USDA-APHIS-PPQ, 2014); but it is not known the population dynamics and hosts of *H. armigera* in these countries.

4. Population dynamics of *H. armigera* and *H. zea*

The population peaks of *Helicoverpa* are generally during flowering and fructification of the crop. Other factors that affect the population are: weather variables, type and variety of crop, transgenic crops with Bt toxins, quantity of fertilizer used in the crop, diversity of the landscape around the crop, date of planting, insecticides group and application regime, migration, diapause of the insect, predators, and parasitoids (Adkisson, 1958; Fitt, 1989; Lukefahr et al., 1971; Maelzer & Zalucki, 1999; Morton et al., 1981; Parajulee et al., 1998; Raulston et al., 1981; Reddy & Manjunatha, 2000; Terry et al., 1987; Wu et al., 2002).

Pheromone and light traps are methods currently in use to monitor adult populations (Guerrero et al., 2014). Adult captures may represent populations from the adjacent crop or from immigrant moths (Allen & Luttrell, 2011). Several researchers report that adult captures of *Helicoverpa* have a correlation with oviposition of eggs on the crops in the United States, Spain, and Australia (Campbell et al., 1992; Izquierdo, 1996; Leonard et al., 1989; Wilson & Morton, 1989). On the other hand, Roltsch & Mayse (1984) did not find any relationship between adult

population and oviposition of eggs in tomato in Arkansas in the period April to July. However, an increase of adults in the traps could be useful to predict an increase of population in the crop and could be a tool for planning of insecticides application (Calero-Toledo, 2007; Hoffmann et al., 1991). Consequently, it is necessary to study the relation between adult, larval, and egg populations for each environment and crops.

For *H. armigera* various researchers found a positive correlation between temperature and adults captured with light traps or larval populations in Australia and Pakistan (Morton et al., 1981; Persson, 1976; Wakil et al., 2010). Differently, Dent & Pawar (1988) found a non-significative relationship between the temperature at night and adult populations captured with pheromone and light traps in Australia. The relationship between precipitation and populations of *H. armigera* adults captured with pheromone and light traps depends on the locality and the season of the year, could be positive, negative, or non-significative according to reports in Australia (Baker et al., 2011; Maelzer & Zalucki, 1999; Morton et al., 1981; Persson, 1976). Wakil et al. (2010) found a negative relationship between relative humidity and larval populations in Pakistan in tomato. On the other hand, Morton et al. (1981) and Dent & Pawar (1988) did not find a relationship with relative humidity and adults captured with light and pheromone traps in Australia. Morton et al. (1981), and Persson (1976) found a negative relationship between wind speed and populations of *H. armigera* adults captured by light traps in Australia. Differently, Dent & Pawar (1988), did not found a relationship between wind speed and adults of *H. armigera* captured with light and pheromone traps in Australia. Morton et al. (1981), and Persson (1976) found a negative relationship between moonlight and adult populations of *H. armigera* adults captured with light traps in Australia, therefore at full moon populations decreased. On the other hand; Dent & Pawar (1988) did not find a relationship with moonlight and *H. armigera* adults captured in light and pheromone traps in Australia.

For *H. zea,* investigations about the correlation with weather variables have been carried in the United States. The temperature has a positive relationship with adult male populations captured in pheromone traps (Parajulee et al., 1998, 2004). The precipitation has a positive or negative relationship with adults captured in pheromone traps, depending on the season and the locality (Parajulee et al., 2004; Slosser et al., 1987). The precipitation influences the moisture of the soil, several researchers found a negative relationship between moisture of the soil and pupal

populations of *H. zea*, in the dry soil the survival of the pupae is higher (Barber & Dicke, 1939; Thomas & Dunnam, 1931; Young & Price, 1968). The wind speed has a negative relationship with adult populations of *H. zea* captured in pheromone traps, and eggs in cotton plants (Nuessly et al., 1991; Parajulee et al., 2004, 1998). In some researches the moonlight had a negative relationship with populations of adults captured with light traps, therefore at full moon populations decreased (Hartstack, 1973; Lopez et al., 1979; Nemec, 1971). On the other hand, Parajulee et al. (1998) report a positive relationship with moonlight and adult populations captured with pheromone traps.

5. *Helicoverpa* and natural hybridization

Natural hybridization is not a rare event in wildlife; in plants 25 % of the species could hybridize with related species; in animals 10 % of the species could crossbreed with close species (Mallet, 2005). In the genus *Heliconius* (Lepidoptera: Nymphalidae) hybridization could occur in 34.8 % of the species (Mallet, 2005). In birds, in the order Anseriformes, 41.6 % of the species could hybridize (Grant & Grant, 1992). Hybridization has been reported in lice (Anoplura), crustaceans of the order Cladocera (Haldane, 1922), fish species (Smith et al, 2003), and mammals (Larsen et al., 2010). In Lepidoptera, hybridization has been reported in the following families: Nymphalidae, Papilionidae, Noctuidae, Pieridae, Notodontidae, Sphingidae, Saturniidae, Geometridae, Lasiocampidae, Erebidae, Psychidae, Tortricidae, Bombycidae, and Lycaenidae (Astaurov, 1969; Aubert et al., 1997; Haldane, 1922; Mavárez et al., 2006; Monti et al., 2001; Porter & Shapiro, 1990; Presgraves, 2002; Sakamoto & Yago, 2017). In some cases hybridization could lead to speciation, usually after several generations, the hybrid was considered a new species, this has been suggested for Darwin's finches, genus *Geospiza* (Lamichhaney et al., 2017), birds of genus *Lepidothrix* (Barrera-Guzmán et al., 2017), butterfly *Heliconius* (Mavárez et al., 2006).

Generation F1 of hybrids usually have less survival and are less fertile than their parental lines (Mallet, 2005), in some cases one sex is sterile or absent (Haldane, 1922). But in other cases the hybrids have advantages over their parental relatives, Grant & Grant (1992) report in birds of genus *Geospiza*, known as Darwin's finches, the hybrids have more survival than their parentals. Hybrids of the plant genus *Helianthus* could live in desserts where their parentals do not grow (Gross & Rieseberg, 2005). Hybrids in the genus *Dacus* (Diptera: Tephritidae) have more survival than their parents at a temperature of 31.5°C (Lewontin & Birch, 1966).

Hardwick (1965) was the first researcher who reported hybridization between *H. armigera* and *H. zea*, under laboratory conditions. Hardwick (1965) obtained hybrids between males *H. zea* and females *H. armigera*, the F1 hybrid generation shared morphological characters of both species in the genitalia, but the first backcross generation with *H. zea* had its genitalia almost indistinguishable from normal *H. zea.* Laster & Sheng (1995) and Laster & Hardee (1995) also reported hybridization of these species in the laboratory. Laster and Sheng (1995), and Laster and Hardee (1995) did not find sterility in any generation of hybrids or in the backcrosses. Other researchers reported hybridization between close species to *H. armigera* and *H. zea*. Hybridization between *H. armigera* and *H. assulta* was documented by Zhao et al. (2005), Wang & Dong (2001), and Tang (2005). Zhao et al. (2005) found some sterile males among the F1 hybrids. Degrugillier & Newman (1993) report hybrids between *H. zea* and *H. assulta.* Hybridization between *Chloridea (Heliothis) virescens* and *Chloridea (Heliothis) subflexa* (Noctuidae: Heliothinae) was reported by Laster (1972), Laster et al. (1988), Cibrian-Tovar & Mitchel (1991), and Teal & Oostendorp, (1993). Laster (1972) found sterile males in the F1 hybrid generation. Laster et al. (1988) did not found evidence of male fertility in the F1 hybrid and backcrosses generations, those results suggest the use of sterile hybrid release to control *C. virescens.* Lepidoptera hybrids have been found in nature: genus *Heliconius* (Mavárez et al., 2006), genus *Papilio* (Papilionidae) (Aubert et al., 1997). In Brazil, possible hybrids between *H. armigera* and *H. zea* have been founded in specimens collected from the field (Anderson et al., 2016, 2018; Leite et al., 2016, 2017; Lopes et al., 2017). In case *H. armigera* and *H. zea* are hybridizing in nature, these hybrids could combine characteristics from both parents, which could give them advantages in the environment.

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CHAPTER II: Population Monitoring of *Helicoverpa armigera* (Hübner) and *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) in Puerto Rico

Abstract

In the period February 2016 to January 2017, 112 yellow bucket Unitraps with a specific commercial lure for *H. armigera* were installed in Puerto Rico. These traps were placed in Guanica, Santa Isabel, Guayama, Isabela, Lajas, Juana Diaz, Añasco, Aguadilla, Sabana Grande, Mayagüez, San Juan, Gurabo, Villalba, and Jayuya. The traps were installed near to 17 plant species. The moths were identified with the morphology of male genitalia. Insects identified as *H. armigera* with morphology were corroborated with Real Time PCR using primers for ITS1 region, and sequencing of COI and Cytb regions. Comparisons of *Helicoverpa* populations between localities and among crops in each locality were done with non-parametric tests. Data of moth capture by a bell pepper farm in Santa Isabel was used to analyze relationship with weather variables. A total of 5122 *Helicoverpa* were captured in 77 traps, and 35 traps did not capture species on the genus. Four *H. armigera* adults were identified, the remaining were *H. zea*. In San Juan, Gurabo, Villalba, and Jayuya any *Helicoverpa* adults were found. The highest numbers of *H. zea* male specimens trapped were in the period February to May. Santa Isabel accumulated the highest number of *H. zea* specimens captured. Comparing crops, only okra in Guanica had higher *H. zea* specimens captured than sunflower. In Santa Isabel, male populations of *H. zea* captured had a significantly negative relationship with maximum temperature in the periods June to September and June to December; and precipitation had a significantly negative relationship in the period June to September. Average temperature and minimum temperature did not result in relationships with adult male populations captured.

1. Introduction

Helicoverpa armigera could affect more than 180 plants, while *H. zea* could attack 123 hosts, both species affect many crops (Cunningham & Zalucki, 2014). *Helicoverpa zea* is distributed in America, while *H. armigera* was distributed in the Old World. *Helicoverpa armigera* was reported for the first time in America in 2013 in Brazil, then was reported in Argentina, Bolivia, Uruguay, Paraguay, and Puerto Rico (Arnemann et al., 2016; Czepak & Albernaz, 2013; Kriticos et al., 2015; Murúa et al., 2014; USDA-APHIS-PPQ, 2014). In September 2014, one adult *H. armigera*

was detected in San Germán, Puerto Rico. In the island, the Plant Protection and Quarantine program (PPQ) of the United States Department of Agriculture detected *H. armigera* near to beans, okra, sorghum, rice and pigeon pea (USDA-APHIS-PPQ, 2014). Resistance to insecticides (Kranthi, et al, 2005) and shorter life cycle (Barbosa et al., 2016) of *H. armigera* than *H. zea* are important factors to take into account to implement an Integrated Pest Management (IPM) for these species.

The population peaks of *Helicoverpa* are generally on flowering and fructification of the crops. Other factors that affect populations are: weather conditions, variety of crop, planting date, transgenic crops, insecticides application, migration, diapause, and natural enemies (Fitt, 1989; Lukefahr et al., 1971; Maelzer & Zalucki, 1999; Morton et al., 1981; Parajulee et al., 1998; Raulston et al., 1981; Terry et al., 1987; Wu et al., 2002).

Traps are used to monitor adult populations, captures in the traps could be from adjacent crops or from immigrant moths (Allen & Luttrell, 2011). Several researchers report that adult captures have a correlation with oviposition of eggs on the crops according to reports in Australia, Spain, and the United States (Campbell et al., 1992; Izquierdo, 1996; Wilson & Morton, 1989). On the other hand, Roltsch & Mayse (1984) did not find any relationship with oviposition of eggs in tomato in Arkansas. However, an increase of adults in the traps could be useful to predict an increase in population in the crop (Hoffmann et al., 1991).

For *H. armigera* and *H. zea*, the literature reports positive correlation between temperature and adults captured with light and pheromone traps or larval populations in Australia, the United States, and Pakistan (Morton et al., 1981; Parajulee et al., 2004, 1998; Persson, 1976; Wakil et al., 2010). The precipitation could have positive or negative relationship with adults captured with light or pheromone traps, depending of the season and the locality according to reports in Australia and the United States (Baker et al., 2011; Maelzer & Zalucki, 1999; Parajulee et al., 2004; Slosser et al., 1987). Wakil et al. (2010) report a negative relationship between relative humidity and larval populations of *H. armigera* in tomato in Pakistan. The wind speed has a negative relationship with adult populations captured with light or pheromone traps in Australia and the United States (Morton et al., 1981; Parajulee et al., 2004, 1998; Persson, 1976). Moonlight could influence adult populations, at full moon some researchers report decreasing of the populations captured with light traps in Australia and the United States (Hartstack, 1973; Lopez et al., 1979; Morton et al., 1981;

Nemec, 1971; Persson, 1976); on the other hand Parajulee et al. (1998) report an increase of adult populations captured with pheromone traps in the United States.

The objectives of the research were: determine the current status of *H. armigera* and *H. zea* populations in Puerto Rico, and analyze the correlation between male adult populations of *Helicoverpa* captured on pheromone traps with weather variables.

2. Materials and Methods

2.1. Quantification of *Helicoverpa* adults with pheromone traps

In the period February 2016 to January 2017, yellow bucket Unitraps (Alpha Scents^{TM}) with lure *IT390* ISCAlure-Armigera specific for *H. armigera* were placed near or between crop lines on flowering or fructification; this pheromone attracts *H. zea* too, thus was quantified populations of both species. The traps were removed at the end of the crop period. A total of 112 traps were installed at 1.5 m from the soil using metal rods. The traps were placed in 14 localities: 39 traps in Guanica, 11 in Santa Isabel, 15 in Isabela, nine in Lajas, seven in Mayagüez, two in Guayama, two in Juana Diaz, two in Añasco, one in Aguadilla, one in Sabana Grande, four in San Juan, nine in Gurabo, five in Villalba, and five in Jayuya. The traps were placed near to 17 plant species: 15 traps near to pigeon pea *(Cajanus cajan* L.), 16 to chili pepper *(Capsicum frutescens* L.), 15 to soybean *(Glycine max* L.), 16 to sunflower *(Helianthus annuus* L.*)*, 12 to corn *(Zea mays* L.), nine to bell pepper (*Capsicum annuum* L.), seven to tomato *(Solanum lycopersicum* L.*)*, five to pumpkin *(Cucurbita moschata* Duchesne), three to bean *(Phaseolus vulgaris* L.), four to cotton (*Gossypium hirsutum* L.), two to okra (*Abelmoschus esculentus* L.), one to sorghum *(Sorghum bicolor* L.), one to rice *(Oryza sativa* L.), one to crotalaria *(Crotalaria* sp); and five traps near to non-hosts of *Helicoverpa*, three to plantain *(Musa paradisiaca* L.), one to Royal palm *(Roystonea borinquena* O. F. Cook*)*, and one to herbaceous weeds. The number of traps per locality and crop is detailed in Table 1. Each locality was monitored according to Table 2.

In Santa Isabel, the traps were placed mainly in two tomato farms, and in a bell pepper farm with continuous production all the year. In Guanica were monitored mainly three farms: one sunflower farm with continuous production all around the year, another farm that harvest soybean and cotton almost all the year; and a vegetable farm that grew tomato, bell pepper, chili pepper, okra, and others. In Isabela were monitored two farms: the substation of the University of Puerto Rico at Isabela, and Tropical Agriculture Research Station (TARS) at Isabela, both farms cultivated

mainly soybean, pigeon pea, and corn during the trapping period. In Lajas the traps were placed in the Agricultural Experimental substation of University of Puerto Rico at Lajas that was growing mainly corn. In Mayagüez two places were monitored: the University of Puerto Rico Mayagüez campus and Alzamora farm of the University of Puerto Rico, the traps were placed mainly near to small experimental blocks of chili pepper and corn. In Guayama the traps were placed only in a private farm with soybean production. In Juana Diaz was monitored the Agricultural Experimental substation of the University of Puerto Rico at Juana Diaz in bean fields. Añasco, Aguadilla, and Sabana Grande were monitored only in February 2016. In Añasco the traps were placed in a private farm with corn and chili pepper. In Aguadilla and Sabana Grande, the traps were placed near to pigeon pea fields in private farms. In San Juan, the traps were monitored in the substation of the University of Puerto Rico at Rio Piedras mainly in small experimental blocks of chili pepper. In Gurabo, the traps were placed mainly near to small fields of chili pepper in the Agricultural Experimental substation of the University of Puerto Rico at Gurabo. In Villalba, the traps were placed near to pigeon pea in private farms. In Jayuya the traps were placed near to small groups of plants of pigeon pea. The traps were reviewed every two or three weeks, most of the times every two weeks. The insects caught in the traps were carried to the laboratory to count populations and identify species of the moths.

Comparisons between localities with the same crop were carried using Mann-Whitney U nonparametric test. Comparisons among crops per each locality were done using Kruskal-Wallis test if the *P*-value was significative a Dunn test was used to compare the medians. The *H. zea* counts in each trap were extrapolated to 15 days prior the analysis. The data was transformed with logarithm+1 to homogenize the variances; before the analysis a Levene test was done to verify the homogeneity of the data. The statistical analysis was done using R software (version 3.3.2): packages FSA (Ogle, 2017) and rcompanion (Mangiafico, 2017).

2.2. Morphological and molecular identification of *Helicoverpa* captured

Helicoverpa males captured had their genitalia extracted following the methods of Brambila (2009): abdomens were placed in vials in groups up to ten per vial, alcohol 70 % was added for less than two minutes, the alcohol was removed, potassium hydroxide 10 % was added, the vials were placed in a water bath at 60°C for one hour, the potassium hydroxide was removed, alcohol 70 %

was added to remove the potassium hydroxide, and the abdomens were placed in alcohol 70 % until dissection. The insects were identified according to Pogue (2004) and Brambila (2009), the main difference between these species is that *H. armigera* has one lobe in the base of the vesica, while *H. zea* has three lobes (Figure 1).

The males classified as *H. armigera* by genitalia morphology were subjected to Real Time PCR using specific primers for the Internal transcribed spacer (ITS)1 region, and sequencing of cytochrome c oxidase I (COI) and Cytochrome b (Cytb) regions. Some sterile *Helicoverpa* males were not possible to identify with morphology because they did not have cornuti (Figure 2A) these insects were identified with Real Time PCR using primers for the ITS1 region.

DNA was extracted from the insect thorax or abdomen with the kit DNeasy Blood and Tissue (QIAGEN, Cat. Number: 69506, Germany), the protocol was slightly modified: per each sample was added 180 µl of buffer ATP, 20 µl of proteinase K, and zirconium beads (2mm), the samples were placed in a Tissue Homogenizer Bullet Blender (NEXT ADVANCE, USA) at speed eight, per four minutes, the samples were centrifuged at 13, 200 rpm for 4-6s, then were placed in a water bath at 56°C per three hours, each 30 minutes the samples were shaken, after the incubation the samples were centrifuged at 13,200 rpm per three minutes. The rest of the extraction was done with the robot QIAcube (QIAGEN, Germany) with the program 'Animal tissues and rodent tails'.

Real Time PCR with ITS1 region, was done with the primers 3373Ha_Hz_ITS1-F (common forward), 3374Ha_ITS1-R *(H. armigera* specific reverse), and 3377Hz_ITS1-R *(H. zea* specific reverse) (Perera et al., 2015). For each reaction of 25 µl was used a mixture: 4.5 µl of water free RNA (QIAGEN), 12.5 µl of SYBR Green PCR Master Mix 2X (QIAGEN, Cat. Number: 204074, Germany), 1 µl of each primer (10 µM), and 5 µl of DNA template. The PCR program was five minutes at 95°C, 35 cycles of 5s at 95°C, and 10s at 60°C. After the PCR reaction a melt analysis was done with the following conditions: ramp from 65°C to 95°C, hold 90s on the 1st step and hold 5s on next steps. Real Time PCR reactions were performed in the equipment Rotor Gene Q (QIAGEN). In the melt analysis *H. armigera* had a peak of fluorescence at 82.8 to 85.5°C, while *H. zea* have a peak at 87.5 to 88.5°C in the equipment used (Figure 8). In these assays, were included positive controls of *H. zea* and *H. armigera* from colonies from the laboratory of the Center for Excellence in Quarantine and Invasive Species of the University of Puerto Rico. In these assays

were included a DNA mix of *H. armigera* and *H. zea*, this mix shows two peaks at similar temperatures than *H. zea* and *H. armigera* (Figure 8) in case one sample is F1 hybrid could have a similar curve than the DNA mix (Perera et al., 2015).

PCR assays with H3Fw and H3Rv primers, that amplify part of the COI region (Arneodo et al., 2015) were performed in this way: 7.75 µl of water, 12.5 µl of Master Mix Promega 2X (Promega, Cat. Number: M7505, USA), 1 µl of each primer (10 µM), 0.75 µl of Bovine Serum Albumin (Sigma, Product #: B8667, USA), and 2 µl of DNA for a total volume of 25 µl. The PCR program was in this manner: five minutes at 95°C, 30 cycles of 30 seconds at 95°C, 30 seconds at 54°C, and 60 seconds at 72°C, and a final extension at 72°C for two minutes. The specific amplified product of ≈812 bp was visualized UV light and cut using a spatula, the gel was removed using the kit DNA Clean & Concentrator (Zymo Research, Cat. Number: D4002, USA).

PCR assays with Cytb-F02 and Cytb-R02 primers, that amplify part of the Cytb region, were prepared according to this protocol: 7.375 µl of water, 12.5 µl of Master Mix Promega 2X (Promega, Cat. Number: M7505, USA), 1.25 µl of each primer (10 µM), 0.625 µl of Bovine Serum Albumin (Sigma, Product #: B8667, USA), and 2 µl of DNA for a total volume of 25 µl. PCR had the following conditions: four minutes at 94°C, 35 cycles of 30 seconds at 94°C, 30 seconds at 50°C, one minute at 72°C, and a final extension at 72°C for ten minutes (Behere et al., 2008). The specific amplified fragment of ≈ 434 bp was purified as reported above for COI fragments. All PCR reactions were performed in a thermal cycler (Applied Biosystems 2070, USA).

Purified amplicons from PCR reactions were submitted for sequencing (Macrogen, Seoul, South Korea). Sequences were edited using CodonCode Aligner Software (version 1.6.3; CodonCode Corporation). The sequences were aligned using the software MUSCLE, version 3.8.31 (Edgar, 2004) and Mesquite, version 3.40 (Maddison, 2018). The sequences were compared with sequences of the GenBank database using the tool BlastN available in the National Center of Biotechnological Information (NCBI) (Johnson et al., 2008). Phylogenetic analyses w inferred using MEGA version 7 (Kumar et al., 2016). The phylogenetic trees were produced using the Maximum Likelihood method based on the Jukes-Cantor model. The bootstrap consensus trees were generated with 1,000 replicates. The initial tree was obtained by applying Neighbor-Join and BioNJ algorithms. A discrete Gamma distribution with invariant sites (G+I) was used to model

evolutionary rate differences among sites. In the phylogenetic trees sequences of *H. zea, H. assulta, Chloridea (Heliothis) virescens,* and *Spodoptera frugiperda* (Lepidoptera: Noctuidae) were included from the GenBank.

2.4. Regression analysis of *Helicoverpa* adult populations and weather variables

Data from *Helicoverpa* counts from Santa Isabel was analyzed against weather variables; this data was obtained from four traps near to bell pepper fields (Figure 3). *Helicoverpa zea* counts for this analysis are in Appendix 2. Weather data from the Agricultural Experimental substation of the University of Puerto Rico at Juana Diaz was obtained (Appendix 3). This AE substation is located 12 km away from the bell pepper farm.

Bell pepper farm has approximately 20 ha, this farm is in front of a tomato farm that is 300 ha approximately (Figure 3A). The tomato farm planted its crop in the period of October to December 2016. Populations of *H. zea* could migrate from tomato to traps in bell pepper fields in the period October-December 2016, for this reason the regression analysis was carried for three periods: one for all time that bell pepper farm was surveyed (June to December 2016), other for bell pepper without tomato adjacent (June to September 2016), and other for bell pepper with tomato adjacent (October to December 2016).

The bell pepper farm grew watermelon *(Citrullus lanatus* Thunb.) all around the year, *Helicoverpa* could attack watermelon, however, cucurbits are not preferred hosts of *Helicoverpa* (Hardwick, 1965). The plots planted with bell pepper and watermelon are detailed in the Figure 3B.

Helicoverpa counts per sampling were extrapolated to 15 days per trap. The counts were transformed with logarithm +1 to normalize the data. In case a trap fell was used the average of the other traps for that trap. The analysis was done with the counts of each trap. Prior analysis normality and homogeneity of data were verified with Shapiro and Levene tests respectively. Linear analysis regressions were done against accumulated precipitation (mm), maximum temperature (°C), minimum temperature (°C), and average temperature (°C). Statistical analysis was done using R software (version 3.3.2).

3. Results and discussion

3.1. Quantification of *Helicoverpa* adults with pheromone traps

During the period February 2016 to January 2017 seventy-seven traps, of 112, captured *Helicoverpa*, while 35 traps did not detect any *Helicoverpa;* only four traps captured *H. armigera.*

In the survey, 5,122 *Helicoverpa* males were captured in the traps. Four adult males were identified as *H. armigera* (Table 3). The remaining 5,118 moths were identified as *H. zea* and originated from ten localities in 13 crops, total captures per locality and per crop is resumed in Table 4 and captures per each trap is detailed in Appendix 1. In San Juan, Gurabo, Villalba, and Jayuya any *Helicoverpa* adults were detected (Figure 4).

Helicoverpa armigera was founded in Guanica, Santa Isabel, and Guayama, (Figure 4). In 2014 during the *Helicoverpa* survey in Puerto Rico made by Plant Protection and Quarantine Program (PPQ), *H. armigera* was detected in San German, Lajas, Yauco, Guanica, and Guayanilla (USDA-APHIS-PPQ, 2014). In both survey, *H. armigera* was detected in the South of Puerto Rico. The production of corn and vegetables such as tomato, peppers, watermelon, and melon is mainly in the south of Puerto Rico (Calero-Toledo, 2007; USDA, 2014). *Helicoverpa armigera* could affect all the crops mentioned (Cunningham & Zalucki, 2014), probably for this reason, the insect has been detected in the south of the island.

In this research, *H. armigera* was detected in pheromone traps near to sunflower, pigeon pea, chili pepper, soybean, and bell pepper. The survey made by PPQ in Puerto Rico detected *H. armigera* in pheromone traps adjacent to fields of beans, okra, sorghum, rice and pigeon pea (USDA-APHIS-PPQ, 2014). In both surveys pheromone traps were used, these traps could attract insects from other crops, and *H. armigera* could migrate up to 1,000 km (Fitt, 1989), therefore the main breeding hosts of *H. armigera* are unknown in the island. In Puerto Rico, the most cultivated hosts that *H. armigera* could affect are soybean 648 ha, corn 410 ha, tomato 345 ha, and pigeon pea 292 ha (USDA, 2014).

In Puerto Rico, *H. armigera* was reported in 2014 (USDA-APHIS-PPQ, 2014). In the current investigation, only 0.08 % (4/5122) of *Helicoverpa* captured were *H. armigera.* In Argentina, where *H. armigera* is an invasive species too, the first detection was in 2013 (Murúa et al., 2014). Murúa et al. (2016) report that the population of *H. gelotopoeon* is apparently higher than *H. armigera* in chickpea and soybean. In Brazil, *H. armigera* entered probably before 2008 (Sosa-Gómez et al., 2016). In this country, apparently *H. armigera* populations are higher than *H. zea* in dicotyledonous hosts, and *H. zea* is predominant above *H. armigera* in corn (Bentivenha et al., 2016; Leite et al., 2014). Hardwick (1965) indicate that *H. zea* prefers corn than any other host.

In general, invasive species have three steps in their invasion process: establishment in new areas, lag period, and spread (Sakai et al., 2001). In the first step the invader must arrive, survive, and establish itself (Allendorf & Lundquist, 2003). The lag period is a time of relative slow growth of the population, this period could be of several years, even decades in some species (Crooks, 2005); the Argentine ant *(Linepithema humile)* (Hymenoptera: Formicidae) had a lag period of 50 years approximately in the United States (Suarez et al., 2001). The spread is a stage of long distance dispersal and replacement of native species by the invasive species (Allendorf & Lundquist, 2003; Sakai et al., 2001). The success of the invader depends on adaptation to new climate, diseases, competition, predation from native species, number of propagules at the arrival, number of introductions, genetic diversity, and type of reproduction (Sakai et al., 2001). In some cases, the population of the invader could collapse due to the factors mentioned (Sakai et al., 2001; Crooks, 2005). It is necessary to continue monitoring populations of *H. armigera* in Puerto Rico to determine the stage of this invasive species.

Total captures of *H. zea* per locality and crop is summarized in Table 4. Most *H, zea* were captured in Santa Isabel (3,821), Guanica (972), and Isabela (222), by traps near to tomato, bell pepper, sunflower, chili pepper, and soybean. The Census of Agriculture 2012, reports that Santa Isabel had 2,417 ha of cropland harvested, including 992 ha of vegetables such as tomato, peppers, and others; Guanica had 915 ha of cropland harvested, including 139 ha of vegetables; Isabela had 392 ha of cropland harvested, including 28 ha of vegetables, and 19 ha of pigeon pea (USDA, 2014). These localities are important agricultural areas in the island, thus they registered the highest numbers of *H. zea.* In these localities, the highest captures of *H. zea* were in the period February to May 2016 (Figure 5) probably for weather conditions (see results and discussion Regression analysis of *Helicoverpa* adults and weather variables). This data is similar than reported by Calero-Toledo (2007) who conducted *H. zea* counts in Puerto Rico (2003-2004) in Santa Isabel and Salinas in corn and tomato, Calero-Toledo (2007) reports the highest peaks of *H. zea* adults in March and May.

Comparisons of localities with the same crops using Mann-Whitney U test are summarized in Table 5. In the period of February to May 2016, tomato in Santa Isabel had higher counts of *H. zea* adult males (median=140.9, *P*-value= 0.0014) than tomato in Guanica (median= 16.0). In Santa

Isabel the traps were placed mainly in a tomato farm with approximately 300 ha, while in Guanica the traps were placed in a farm with approximately 3 ha of tomato; for this reason, is possible to speculate that Santa Isabel had higher *H. zea* counts than Guanica. In the period of May to September, soybean in Guanica (median = 1.46) had no differences with soybean in Isabela (median= 0). In Guanica and Isabela the traps were placed in areas less than one hectare of soybean fields.

In Santa Isabel, in the period from February to May 2016, four traps adjacent to tomato fields registered the highest *H. zea* counts of all the survey (2,061). In this locality seven traps were placed near to bell pepper which registered 1, 760 *H. zea*, the highest *H. zea* peaks were between May to June (Figure 5). The Census of Agriculture 2012, reports that Puerto Rico had 2,757 ha of vegetables such as tomato, peppers, and others; the locality with more cropland harvested with vegetables in the island is Santa Isabel (992 ha) (USDA, 21014), probably for this reason this locality registered the highest *H. zea* counts in the traps over the other localities monitored in this survey.

In Guanica, 16 traps were located in sunflower fields, these captured 216 *H. zea*, in this crop the highest *H. zea* counts were in February, two traps in bell pepper captured 131 *H. zea* with a peak in March, four traps in soybean registered 52 *H. zea* with a peak in April; three traps in tomato captured 151 *H. zea* with a peak in May, four traps in chili pepper captured 172 *H. zea* with a peak in June, four traps in cotton registered 95 *H. zea* with a peak in September, four traps in pumpkin captured 42 *H. zea* with a low peak in October, and two traps in okra registered 103 *H. zea* with a peak in December (Table 4, Figure 6). The Census of Agriculture 2012, reports that Guanica had 139 ha of vegetables (USDA, 2014). In this locality the diversity of plants that *Helicoverpa* could be hosted is high; when one crop is over *Helicoverpa* adults could fly to other hosts, this insect could migrate up 1,000 km (Fitt, 1989; Westbrook & López, 2010), probably for this reason in this locality *H. zea* had peaks during all the survey, however the highest populations were in the period February to May 2016. Comparisons among crops in Guanica with Kruskal-Wallis test is summarized in Table 6. This analysis was done in three periods: February to May 2016, May to July 2016, and August 2016 and January 2017. Only in the period August 2016 to January 2017 the counts of *H. zea* were significantly different, okra (median= 4.60, *P*-value= 0.0001) had higher *H. zea* counts than sunflower (median= 0), and similar counts than cotton (median= 2.32), and pumpkin (median= 1.86); cotton and pumpkin had similar counts than sunflower. This data suggests that *H. zea* prefers

okra over sunflower, however, is necessary to monitor populations of eggs and larvae to affirm this statement. In the periods February to May 2016 and May to July 2016 the crops monitored, sunflower, tomato, bell pepper, chili pepper, soybean, and cotton registered similar counts of *H. zea* adults.

In Isabela, eight traps were placed near to soybean fields, these registered 91 *H. zea*, one trap in pigeon pea captured 22 *H. zea*, three traps in corn registered 16 *H. zea*, soybean, pigeon pea, and corn registered the highest *H. zea* populations in the beginning of February, one trap in crotalaria captured 74 *H. zea* with a peak in May, one trap in sorghum registered 3 *H. zea* with a low peak in July, and one trap in bean registered 16 *H. zea* with a low peak in September (Table 4, Figure 7). The Census of Agriculture 2012, reports that Isabela had 28 ha of vegetables, and 19 ha of pigeon pea (USDA, 2014). Comparisons among crops with Kruskal-Wallis in this locality is presented in Table 7; this test was done for three periods: February to March 2016, May to June 2016, and August to October 2016. No differences were detected among the crops monitored (soybean, pigeon pea, corn, crotalaria, bean, and sorghum) during the 3 periods. Hardwick (1965) reports that *H. zea* prefers corn than any other host, in the traps near to corn in Isabela was not observed this situation, however larval populations were not monitored to confirm this analysis.

In Juana Diaz, two traps were placed near to bean fields, these captured 29 *H. zea*, from which 24 were registered in March. Martorell (1976) reports that *H. zea* is a minor pest in bean in Puerto Rico, probably for this reason the populations were relatively low in this crop.

In Lajas, five traps were placed in fields of corn which captured nine *H. zea*, two traps in chili pepper registered 12 *H. zea*, one trap in rice and one trap in pumpkin did not capture any *Helicoverpa*. Even though corn is the preferred host of *H. zea*, the captures in the traps were low in Lajas. Other factors could affect populations of *Helicoverpa* such as weather conditions, natural enemies, applications of pesticides (Fitt, 1989; Parajulee et al., 2004).

Two traps were placed near to soybean in Guayama, these captured 17 *H. zea*. This locality was monitored in the period July to November 2016, during these months the populations were low probably for weather conditions (see results and discussion Regression analysis of *Helicoverpa* adults and weather variables).

In Mayagüez, three traps were placed near to corn, three to chili pepper, and one to pigeon pea, from these only two traps near to corn captured three *H. zea*. The plots monitored in this locality were small experimental blocks; Mayagüez is an urban area that has been losing cropland (López et al., 2001) probably for these reasons the captures were low.

In Añasco, one trap was placed to field of corn, and one to chili pepper, these traps captured 16 and 11 *H. zea* respectively. The only trap in Aguadilla captured four *H. zea* near to pigeon pea. In Sabana Grande, one trap was placed near to pigeon pea that captured two *H. zea*. Añasco, Aguadilla, and Sabana Grande were monitored only in February 2016, probably for the short time of monitoring in these localities the captures were relatively low.

Helicoverpa was not captured in Villalba in five traps placed near to pigeon pea. The pods of pigeon pea could be affected by other insects that could compete with *H. zea,* Martorell (1976) indicates that *Chloridea (Heliothis) virescens* is an important pest in the pods of pigeon pea in Puerto Rico. Also, other factors could affect the populations of *H. zea* such as weather conditions or natural enemies (Fitt, 1989; Parajulee et al., 2004).

In San Juan, two traps were placed near to small blocks of chili pepper, one trap to royal palm, and one to weeds, the two last are non-host of *Helicoverpa.* Any *Helicoverpa* was captured in this locality. San Juan is the main urban area of Puerto Rico and has been losing cropland (López et al., 2001), probably for these reasons the traps did not capture any *Helicoverpa.*

In Gurabo were placed four traps near to chili pepper, one trap to pigeon pea, one trap to soybean, and three traps to plantain that is non-host of *Helicoverpa*, any of the traps in Gurabo registered *Helicoverpa*. In Jayuya five traps were placed to plants of pigeon pea, in this locality any *Helicoverpa* was captured by the traps. In Gurabo and Jayuya *Helicoverpa* was not detected probably because the traps were adjacent to small plantations. Weather conditions and natural enemies could affect the populations of *Helicoverpa* (Fitt, 1989; Parajulee et al., 2004).

3.2. Morphological and molecular identification of *Helicoverpa* captured

In total 5,122 *Helicoverpa* were captured, with morphological tools four insects were identified as *H. armigera,* 5,075 as *H. zea,* and 43 (0.84 %) were sterile males that were not possible to identify with morphology. In Real Time PCR assays in the melt analysis, the samples of *H.*

armigera from the traps had peaks of fluorescence at 84.5°C equal as *H. armigera* positive control (Figure 8A).

The sterile males of *Helicoverpa* had peaks of fluorescence at 87.5 to 88.0°C the similar as *H. zea* positive control (Figure 8B) therefore, these sterile males were *H. zea*. The sterile males had the aedeagus without cornuti and constricted apex (Figure 2A); the valves were different than normal *H. zea*, see Figure 6B Chapter III, the valves of sterile males were more pointed and without corona (Figure 2B). Balbi et al. (2017) and Hardwick (1970) report *H. zea* with similar morphology as sterile males founded in the current research, these "aberrant" *H. zea* were identified as *Heliothis stombleri* by other researchers, however Balbi et al. (2017) demonstrated that *H. stombleri* is clustered together with *H. zea* in topological trees using mitochondrial and nuclear genes.

In total were identified four *H. armigera* and 5,118 *H. zea*. The sequences of *H. armigera* from the captures, with the regions COI and Cytb, coincided with *H. armigera* in the comparison with the tool BlastN. The phylogenetic trees with both regions, show that *H. armigera* formed a monophyletic clade, and *H. zea* is a sister taxa to *H. armigera* (Figures 9 and 10), as reported by other studies (Anderson et al., 2016; Cho et al., 2008). The phylogenetic tree with the COI region shows that *H. armigera* from Puerto Rico sample t6 collected in Guayama is closer to the sequence KP984524.1 *H. armigera* from Argentina (Arneodo et al., 2015), the other *H. armigera* from Puerto Rico samples t1, t2, and t3 do not have close relationships to *H. armigera* from other parts of the world (Figure 9). The phylogenetic tree with the Cytb region shows that *H. armigera* from Puerto Rico sample t6 is closer to *H. armigera* sequences EF410021.1, EF410023.1, and EF410024.1; these sequences are haplotypes founded in Asia, Africa, Europe, Brazil, and Australia (Supplementary Table 2 from Tay et al., 2017), the other *H. armigera* from Puerto Rico samples t1, t2, and t3 do not have closer relationships with other *H. armigera* (Figure 10).

3.3. Regression analysis of *Helicoverpa* adults and weather variables

Captures in the bell pepper farm registered only one *H. armigera*, consequently, this analysis was done only for *H. zea*. The relationship between *H. zea* populations and weather variables is summarized in Table 8.

In the entire period that this farm was monitored, June to December 2016, only the maximum temperature had a negative relationship with *H. zea* populations (r= -0.41, *P*-value= 0.0019). In contrast Parajulee et al. (1998, 2004) report a positive relationship with *H. zea* captures and maximum temperature. However, investigations of Parajulee et al. (1998, 2004) were conducted in Texas with different weather conditions than Puerto Rico. The maximum temperatures reported by Parajulee et al. (1998) had an average of 24°C (8 to 38.2°C), while in the current investigation the maximum temperatures had an average of 33.7 °C (32.8 to 34.7). Fye & McAda (1972) report that temperatures of 30 and 33 °C decreased the fecundity in *H. zea.* Jones et al. (1978) indicate that temperature at 30°C on larvae and pupae stages reduced the viability and fertility of *H. zea*. The other variables such as precipitation, minimum temperature, and average temperature did not have any influence on the populations.

In the period June to September 2016, when the pepper farm had no tomato plants adjacent to the farm, the precipitation had a significant negative relationship with *H. zea* captures (r= -0.57, *P*-value= 0.007). Slosser et al. (1987), in Texas, report a significant negative relationship between precipitation and *H. zea* populations in May, but a significant positive relationship in April, July, and September. Parajulee et al. (2004), in Texas, report a significant positive relationship between *H. zea* and precipitation. Precipitation affects directly the moisture of the soil, several authors indicate that in moist soils the survival of *H, zea* pupae decrease in Virginia, New Jersey, Connecticut, Massachusetts, and Oklahoma (Barber & Dicke, 1939; Young & Price, 1968). Nuessly et al. (1991) indicate that precipitation dislodges *H. zea* eggs from the plants under laboratory conditions with simulated weather variables. The maximum temperature had a significantly negative relationship (r=-0.53, *P*-value=0.0019) to captured specimens, and this situation was discussed previously.

In the period October to December 2016, when the bell pepper farm had tomato plants adjacent to the farm, any weather variable had a significant relationship with *H. zea* adult populations. Martin et al. (1976), in Florida, indicate that larvae of *H. zea* affect more tomato than bell pepper under field conditions. In the current research probably in tomato fields adjacent to bell pepper farm, the larval populations were bigger, as a result, the adults could migrate from tomato to

traps installed in bell pepper. In the period October to December 2016, *H. zea* populations tended to increase, probably for the migrating *H. zea* adults from the tomato farm.

4. Conclusions

Using the commercial lures, only four *H. armigera* male specimens were detected during the period of February 2016 to January 2017, from a total of 5,122 specimens collected of *Helicoverpa*, the rest of insects were *H. zea.* This suggests that *H. zea* is the predominant species in Puerto Rico or the available lures are in same way bias toward *H. zea*, or the combination of both. The highest populations of *H. zea* were trapped between February to May. In Santa Isabel was registered the highest population captured of *H. zea.* In a bell pepper farm in Santa Isabel during the period of June to December 2016, only the maximum temperature had a negative relationship with *H. zea* populations. In the period of June to September 2016, without tomato adjacent to the bell pepper farm, the maximum temperature and precipitation had negative relationships with *H. zea* populations. In the period of October to December 2016 in this farm, any weather variable had a significant relationship with *H. zea* populations.

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Table 1: Location and number of *Helicoverpa* yellow bucket Unitraps in different localities and crops in Puerto Rico. February 2016 to January 2017.

Locality/ crop	Pigeon pea	Chili pepper	Soybean	Sunflower	Corn	Bell pepper	Tomato	Pumpkin	Bean	Cotton	Okra	Sorghum	Rice	Crotalaria	Plantain*	Royal palm*	Weeds *	Traps/ locality
Guanica		4	4	16		2	3	4		4	2							39
Santa Isabel						7	4											11
Isabela	1		8		3				1			1		1				15
Lajas		2			5			1					1					9
Mayagüez	1	3			3													7
Guayama			2															2
Juana Diaz									2									2
Añasco		1			1													2
Aguadilla	1																	1
Sabana Grande	1																	1
San Juan		2														1	1	4
Gurabo	1	4	1												3			9
Villalba	5																	5
Jayuya	5																	5
Traps/ crop	15	16	15	16	12	9	7	5	3	4	2	1	1	1	3	1	1	112

* Non-hosts of *Helicoverpa*

Table 2: Monitoring periods for *Helicoverpa* in 14 localities in Puerto Rico. February 2016 to January 2017.

	February	March	April	May	June	July	August	September	October	November	December	January
Guanica	■	■	■	■	■	■	■	■	■	■	■	■
Santa Isabel	■	■	■	■	■	■	■	■	■	■	■	■
Isabela	■	■	■	■	■	■	■	■	■	■	■	■
Lajas		■	■	■	■	■	■	■	■	■	■	■
Mayagüez	■	■	■	■	■	■	■	■	■	■	■	■
Guayama						■	■	■	■	■		
Juana Diaz	■	■	■			■	■	■	■	■		
Añasco	■											
Aguadilla	■											
Sabana Grande	■											
San Juan			■	■	■	■	■	■	■	■	■	
Gurabo			■	■	■	■	■	■	■	■	■	
Villalba								■	■	■	■	■
Jayuya								■	■	■	■	■

Table 3: Traps location, date, and crops near to *Helicoverpa armigera* detections. February 2016 to January 2017.

Sample	Locality	Longitude	Latitude	Date	Crop(s) near to the trap
t2	Guanica	18.00935	-66.89254	Aug 24, 2016	Sunflower, pigeon pea
t3	Guanica	17.98633	-66.90435	Aug 24, 2016	Chili pepper
t6	Guayama	17.98781	-66.21546	Aug 24, 2016	Soybean
t1	Santa Isabel	17.98494	-66.42641	Sep 21, 2016	Bell pepper

Table 4: Total absolute of adult males of *Helicoverpa zea* captured per crop and locality. February 2016 to January 2017.

Locality/ crop	Tomato	Bell pepper	Sunflower	Chili pepper	Soybean	Okra	Cotton	Crotalaria	Bean	Corn	Pumpkin	Pigeon pea	Sorghum	Total/ locality
Santa Isabel	2061	1760												3821
Guanica	161	131	216	172	52	103	95				42			972
Isabela					91			74	16	16		22	3	222
Juana Diaz									29					29
Añasco				11						16				27
Lajas				12						9				21
Guayama					17									17
Aguadilla												4		4
Mayagüez										3				3
Sabana Grande												2		2
Total/ crop	2222	1891	216	195	160	103	95	74	45	44	42	28	3	5118

Table 5: Mann-Whitney U test for comparisons *Helicoverpa zea* captured on localities in two seasons. February to September 2016.

Season	Crop	Locality	n[1]	Non-transformed data		Transformed data: Log +1		*P*-value
				Min-Max	Median	Min-Max	Median	
February-May	Tomato	Santa Isabel	13	15-496	140.90	1.2-2.70	2.15	0.0014
		Guanica	6	1-40	16.00	0.3-1.61	1.16	
May-September	Soybean	Isabela	10	0-19	0.00	0-1.3	0.00	0.3530
		Guanica	10	0-7	1.46	0-0.9	0.38	

1: n= number of observations

Table 6: Kruskal-Wallis test for comparisons *Helicoverpa zea* captured among crops in Guanica, in three seasons. February 2016 to January 2017.

Season	Crop	n[1]	Non-transformed data		Transformed data: Log +1		*P*-value	Dunn test *
			Min-Max	Median	Min-Max	Median		
February-May, 2016	Sunflower	4	14-50	31.50	1.18-1.71	1.40	0.8065	-
	Tomato	5	2-40	17.88	0.48-1.61	0.90		
	Bell pepper	6	2-36	31.00	0.48-1.54	1.25		
May- July, 2016	Chili pepper	19	0-31	2.80	0-1.5	0.58	0.1778	-
	Sunflower	16	0-29	3.00	0-1.48	0.60		
	Soybean	7	0-7	0.00	0-0.9	0.00		
	Cotton	7	0-12	1.00	0-1.11	0.30		
August 2016-January 2017	Okra	19	0-14	4.60	0-1.18	0.75	0.0001	A
	Cotton	10	0-12	2.32	0-1.12	0.52		A B
	Pumpkin	21	0-6	1.89	0-0.84	0.46		A B
	Sunflower	36	0-10	0.00	0-1.04	0.00		B

1: Number of observations

* Crops with the same letter are not significantly different.

Table 7: Kruskal-Wallis test for comparisons *Helicoverpa zea* among crops in Isabela, in three growing seasons. February 2016 to October 2016.

Season	Crop	n[1]	Non-transformed data		Transformed data: Log +1		*P*-value
			Min-Max	Median	Min-Max	Median	
February- March, 2016	Soybean	3	11-34	16.00	1.08-1.54	1.23	0.0880
	Pigeon pea	3	3-14	10.00	0.6-1.11	1.04	
	Corn	3	0-10	8.00	0-1.04	0.95	
May- June, 2016	Crotalaria	3	0-39	2.10	0-1.6	0.49	0.6579
	Soybean	3	0-19	1.40	0-1.3	0.38	
August – October, 2016	Soybean	8	0-3	0.00	0-0.6	0.00	0.1006
	Bean	4	0-7	2.25	0-0.9	0.71	
	Sorghum	4	0-1	0.00	0-0.3	0.00	

1: n= number of observations

Table 8: Regression analysis between *Helicoverpa zea* captured during two weeks and weather variables. Santa Isabel- PR, bell pepper farm. Shown three periods: complete sampling June to December 2016, June to September 2016, and October to December 2016.

Season	June – December		June - September (without tomato adjacent)		October- December (tomato adjacent)	
Weather factor	Correlation coefficient	*P*-value	Correlation coefficient	*P*-value	Correlation coefficient	*P*-value
Precipitation (mm)	-0.08ns	0.574	-0.57**	0.0007	0.31ns	0.1468
Max. temp (°C)	-0.41**	0.0019	-0.53**	0.0019	-0.25ns	0.2472
Min. temp (°C)	-0.07ns	0.5982	0.09ns	0.6227	-0.29ns	0.1724
Avg. temp (°C)	-0.17ns	0.2205	-0.26ns	0.1432	-0.28ns	0.1922

ns: Non-significant differences

**: Significant differences

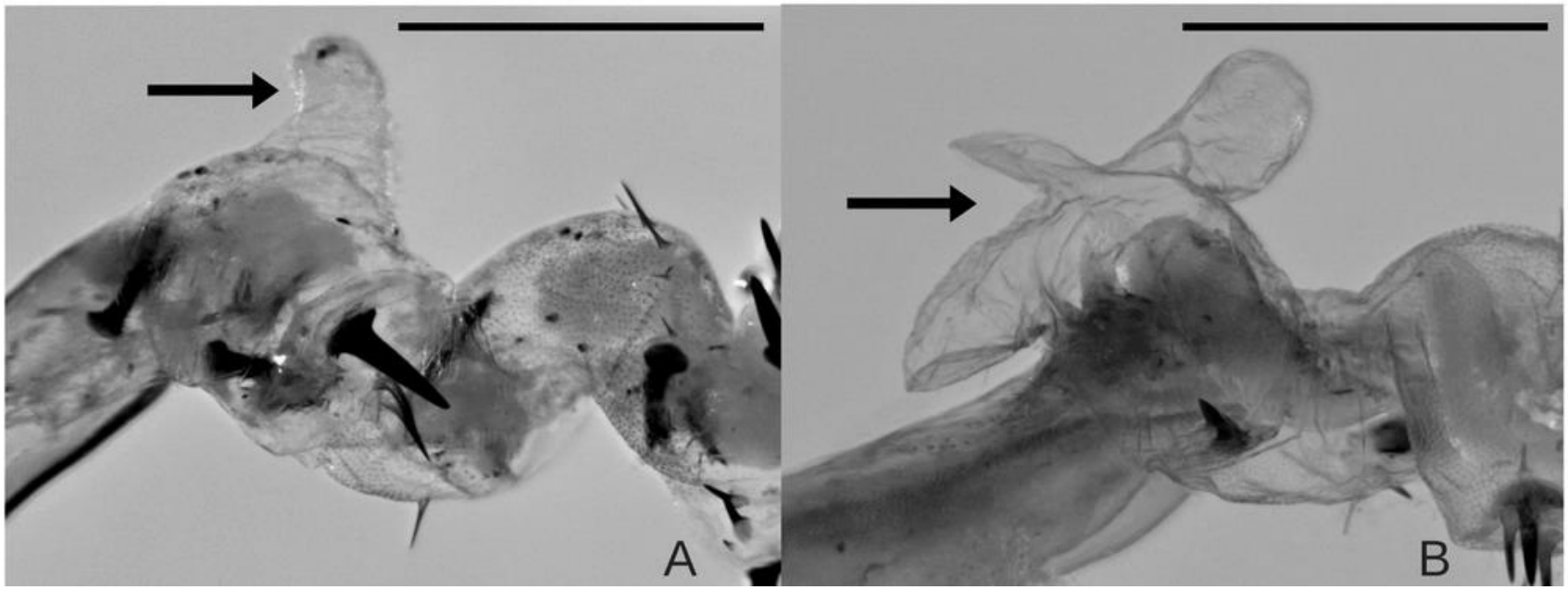

Figure 1: Number of lobes on the base of the vesica, arrows indicate the lobes. **(A)** *Helicoverpa armigera* with one lobe. **(B)** *Helicoverpa zea* with three lobes. Length bar = 1 mm.

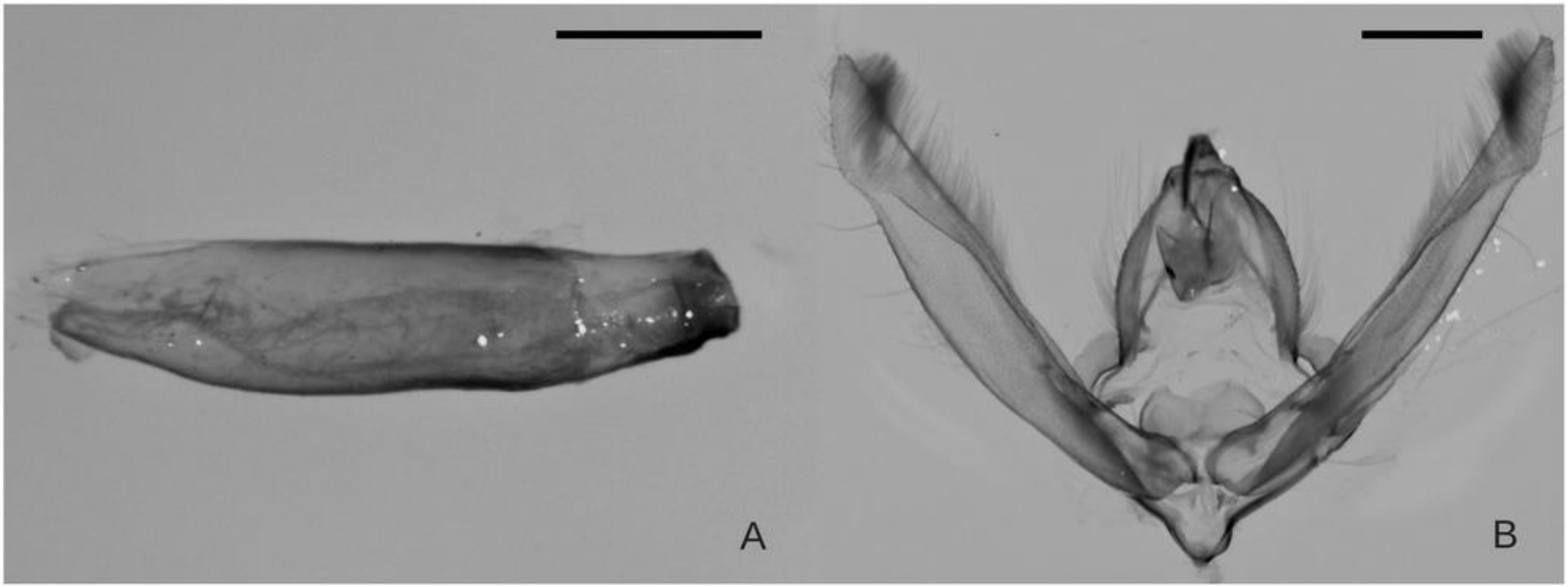

Figure 2: Genitalia structures of sterile males of *Helicoverpa zea*. **(A)** Aedeagus. **(B)** Valves. Length of bar= 1mm.

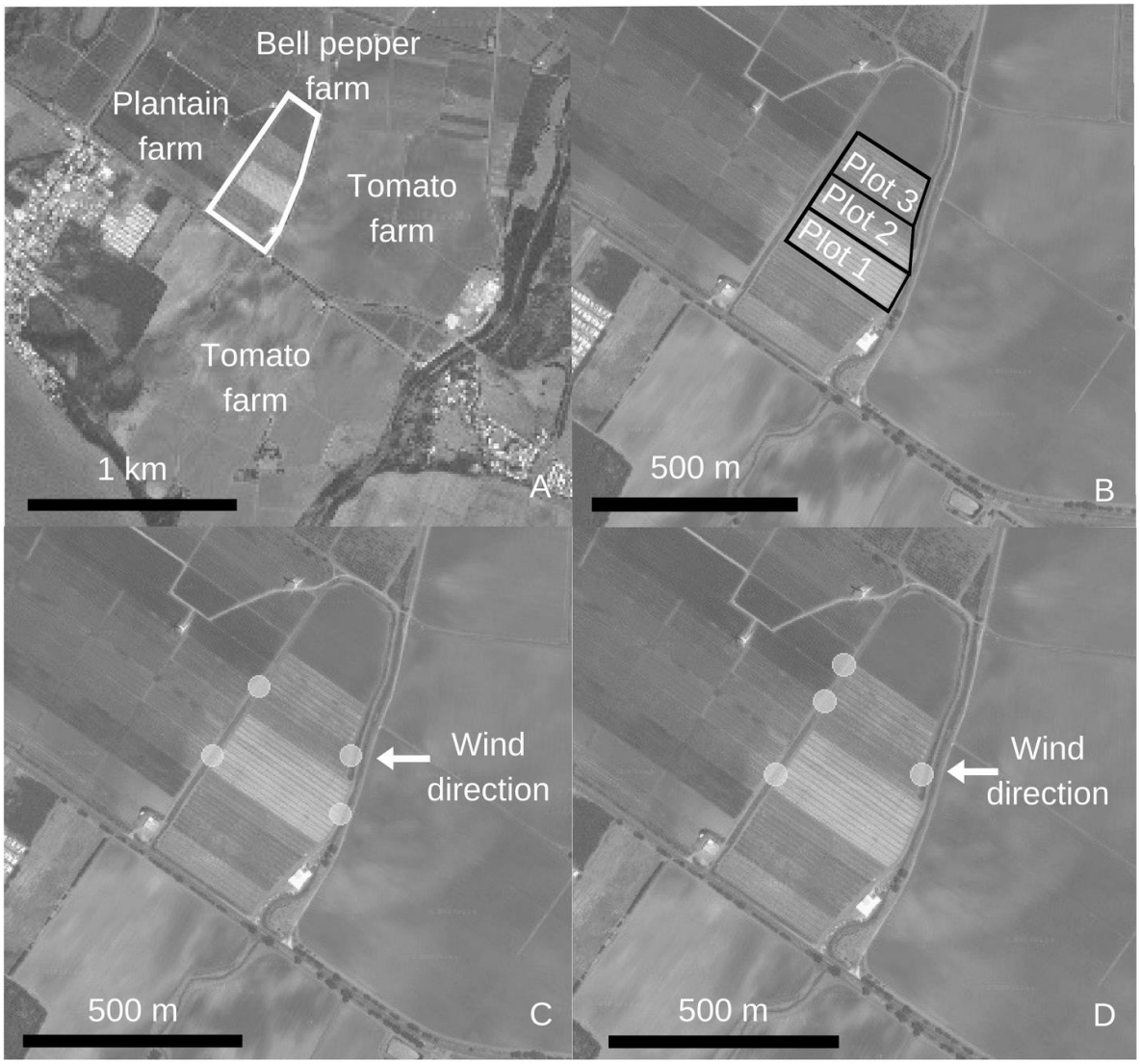

Figure 3: Map of bell pepper farm, Santa Isabel. Numbers of *Helicoverpa* captured were used for regression analysis with weather variables, June to December 2016. **(A)** Surroundings of the farm, bell pepper field delimited by the polygon. **(B)** Crops planted in the period monitored; plot 1: bell pepper June to July, and watermelon September to December; plot 2: bell pepper June to September, and watermelon November to December, plot 3: bell pepper July to October and November to December. **(C)** Traps location (grey dots) in the period June to July. **(D)** Traps in the period August to December. (Source map: Google maps)

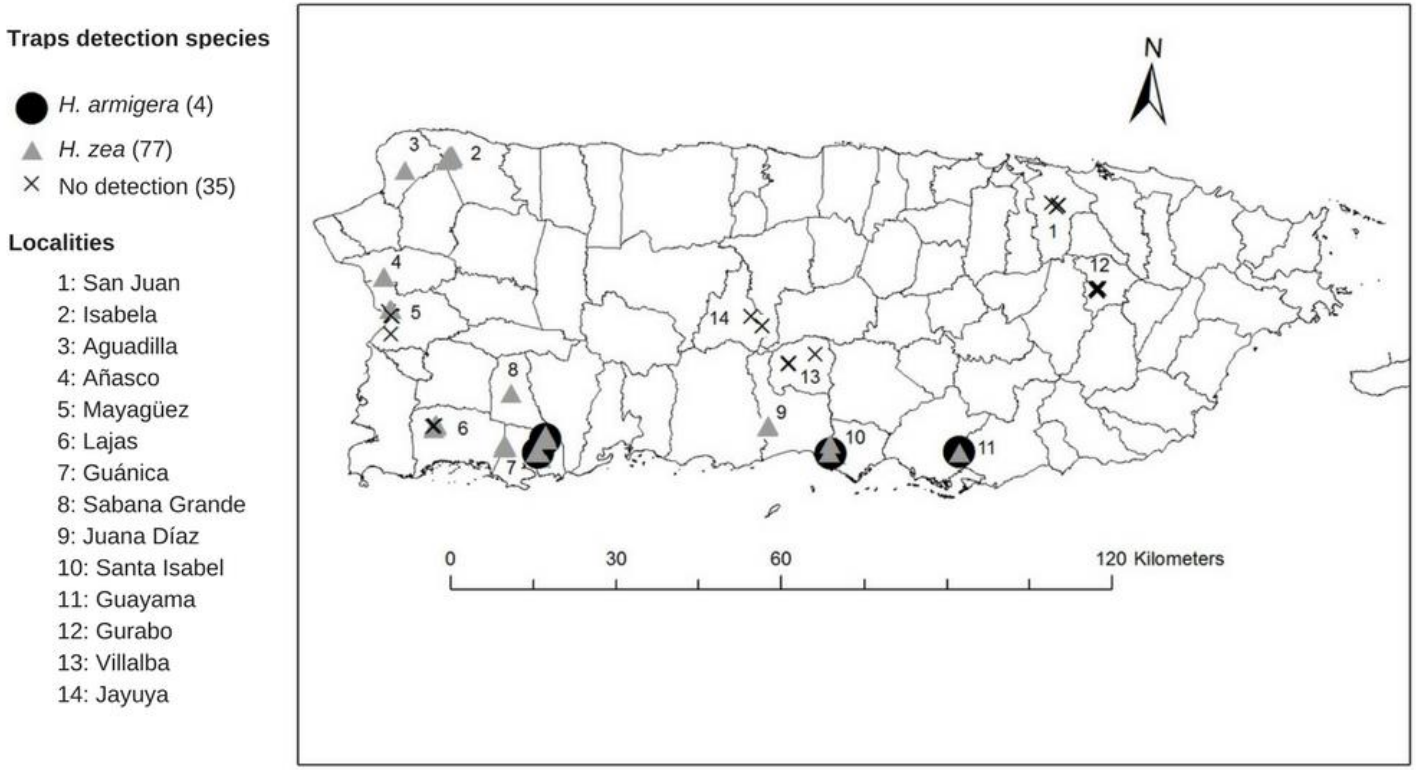

Figure 4: Traps location and *Helicoverpa* spp. captured in Puerto Rico. Number of traps is between parentheses. February 2016 to January 2017.

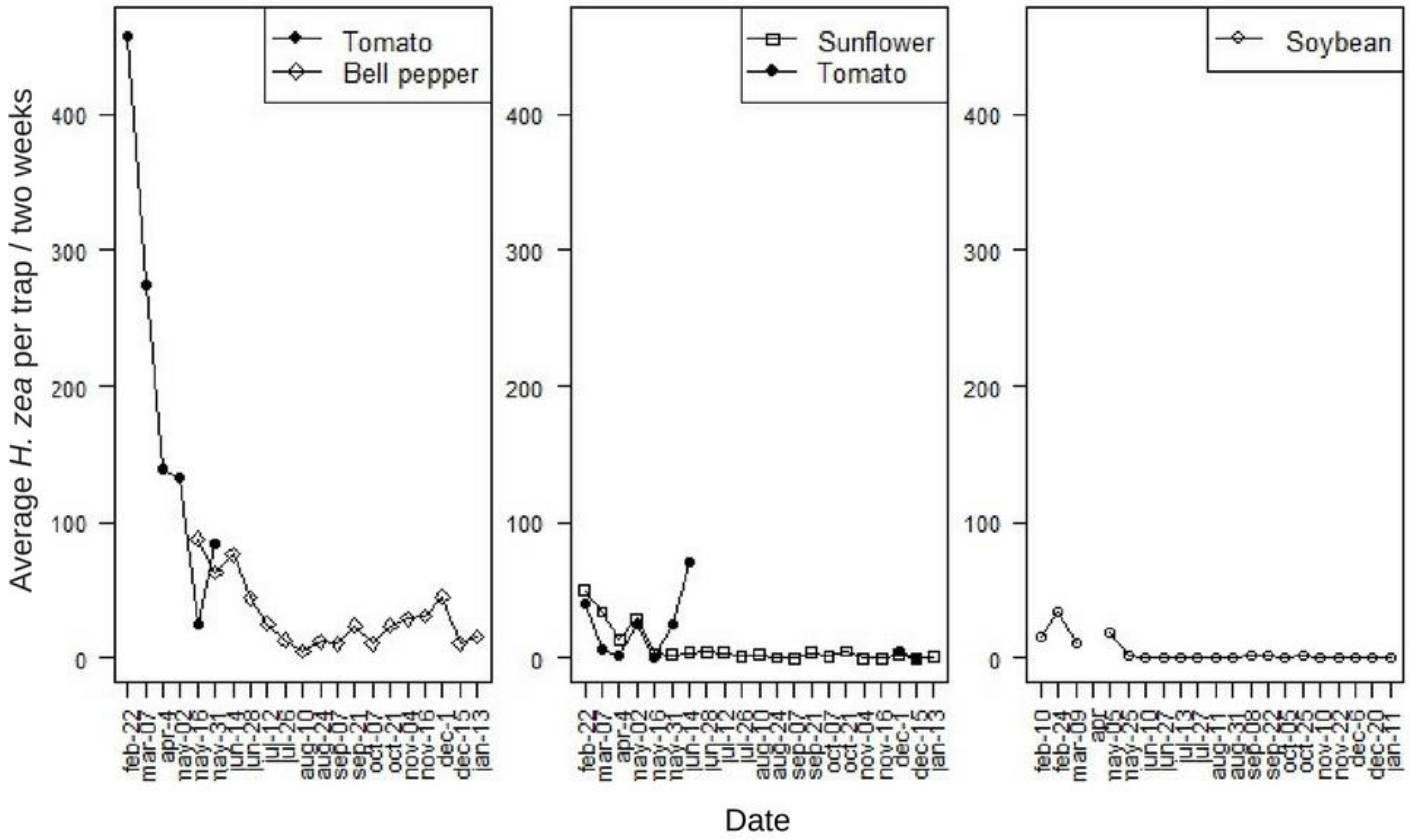

Figure 5: Average number *H. zea* captured per trap (two weeks period) in three localities and crops with higher counts. Left to right: Santa Isabel, Guanica, and Isabela. February 2016 to January 2017.

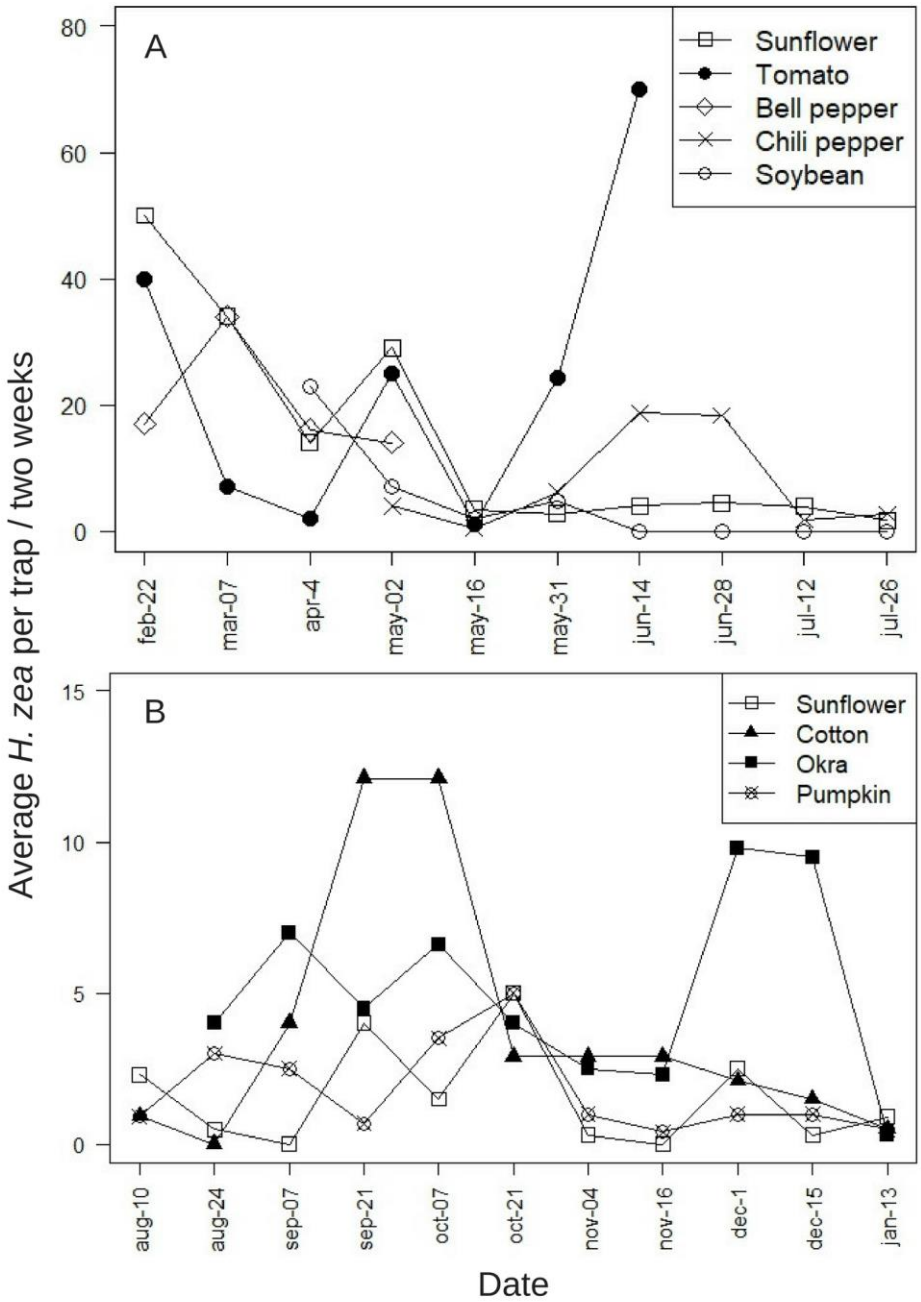

Figure 6: Average number *H. zea* captured per trap (two weeks period) in Guanica, in all crops monitored. February 2016 to January 2017. **(A)** February to July 2016. **(B)** August 2016 to January 2017.

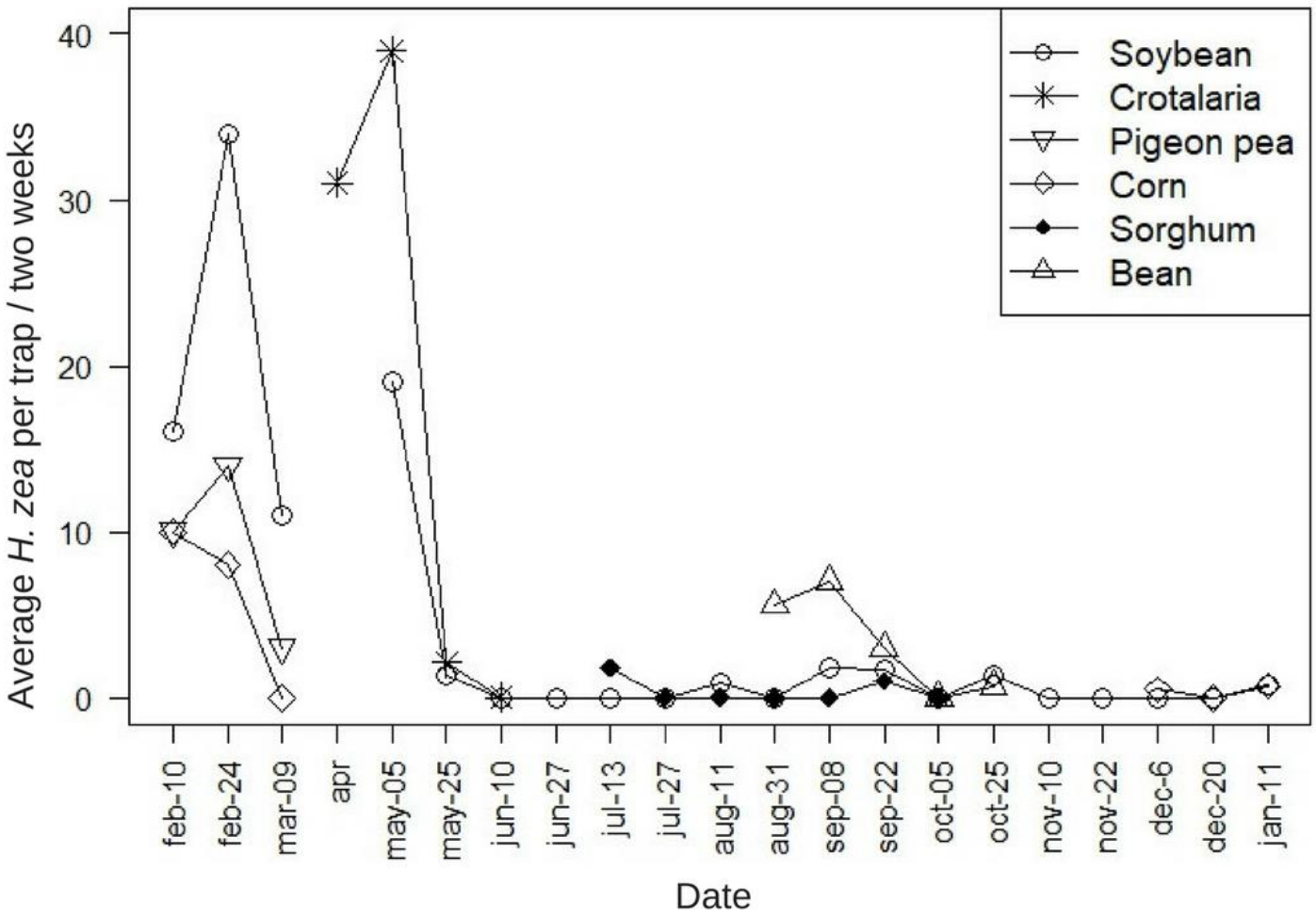

Figure 7: Average number *H. zea* captured per trap (two weeks period) in Isabela, in all crops monitored. February 2016 to January 2017.

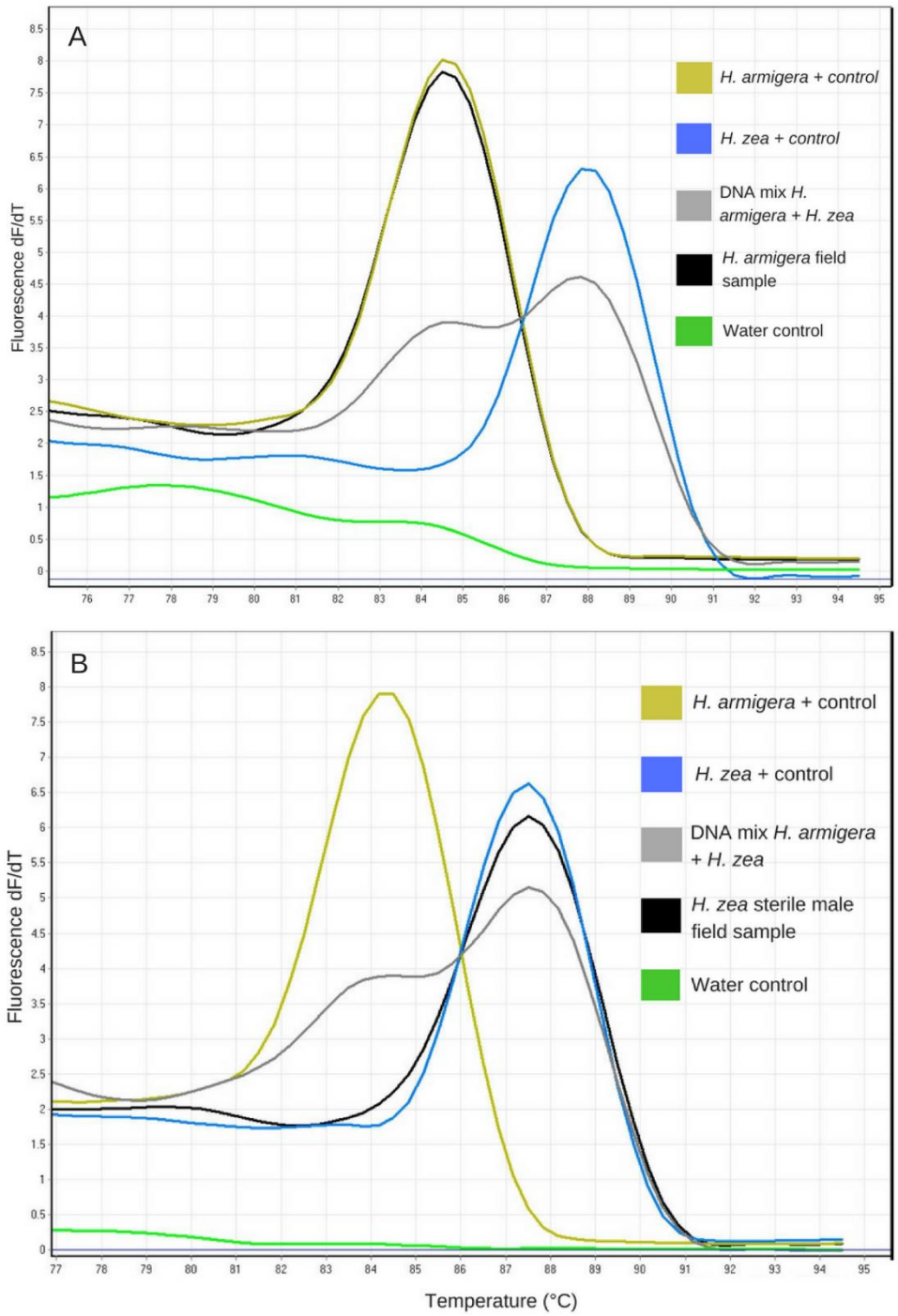

Figure 8: Melting curve analysis for Real Time PCR products from primers ITS1 specific for *Helicoverpa armigera* and *Helicoverpa zea* (Perera et al., 2015). **(A)** Identification of *H. armigera* captured from the field in bucket traps, all *H. armigera* samples had a similar peak like the black curve. **(B)** Identification of *H. zea* sterile males captured from the field in bucket traps, all *H. zea* samples had a similar peak like the black curve.

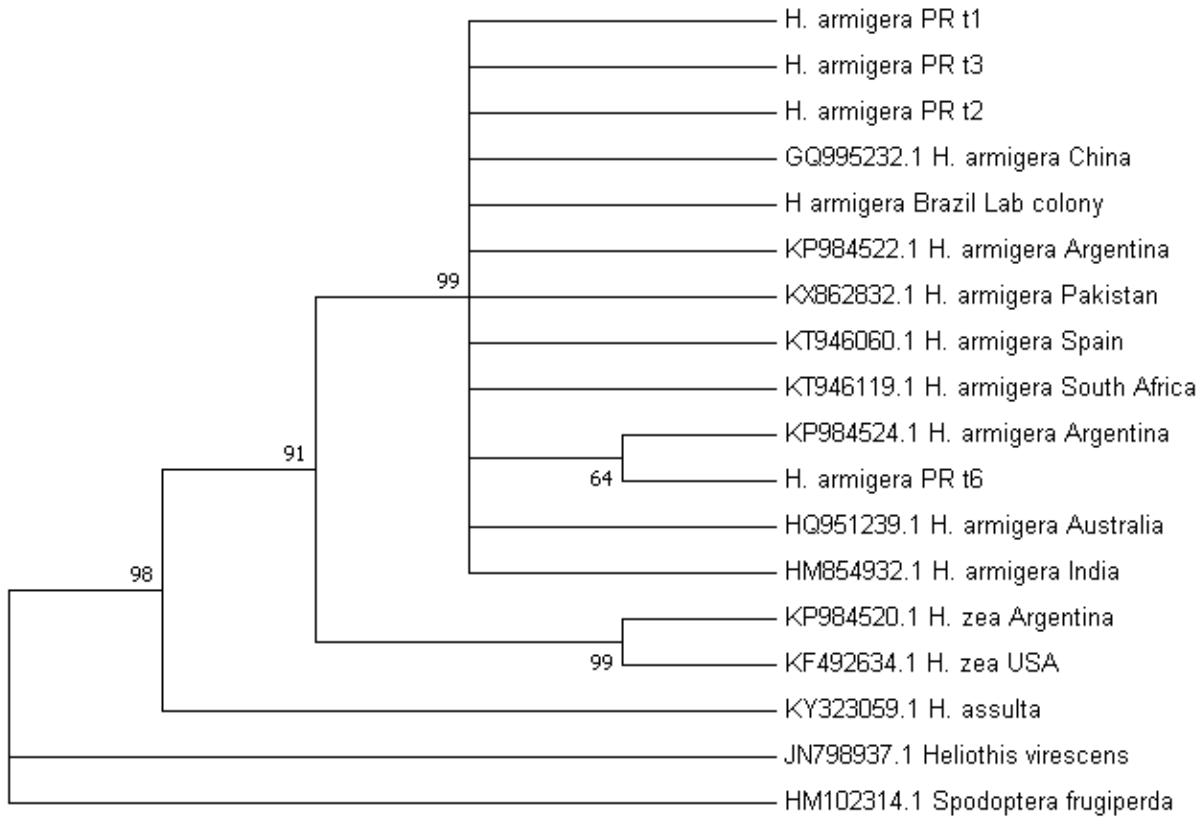

Figure 9: Molecular phylogenetic analysis with Cytochrome Oxidase I region of *Helicoverpa armigera* captured in the field, Puerto Rico. Phylogenetic tree inferred by using the Maximum Likelihood method based on the Jukes-Cantor model. Number on the nodes represent bootstrap percentages (1000 replicates). Accession numbers for sequences from GenBank are shown.

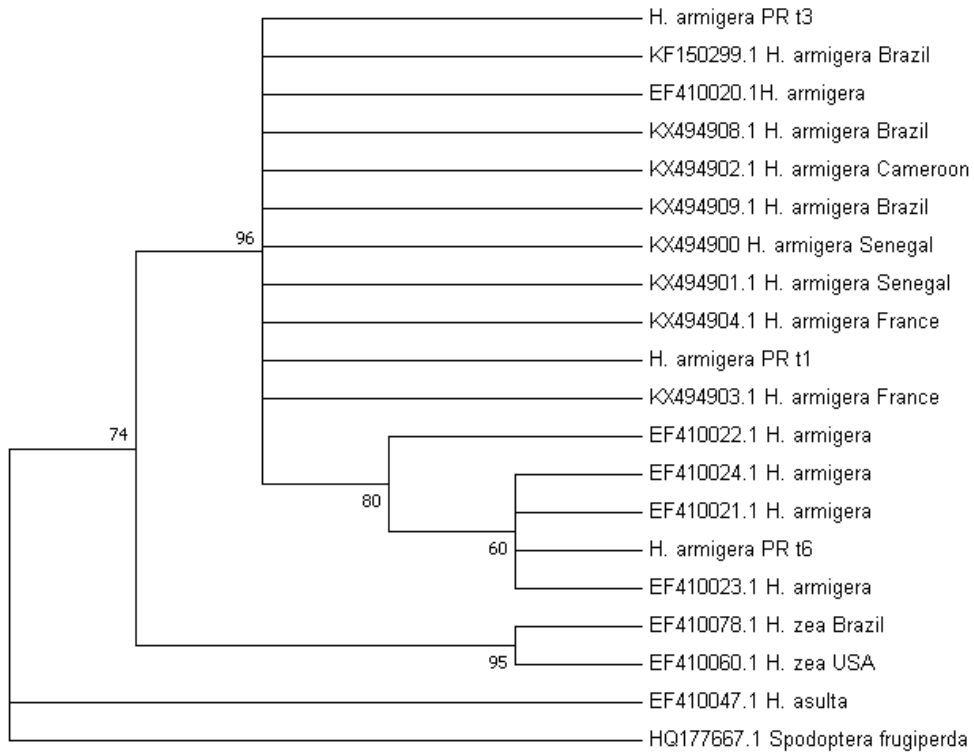

Figure 10: Molecular phylogenetic analysis based on Cytochrome b region of *Helicoverpa armigera* captured in the field, Puerto Rico. Phylogenetic tree inferred by using the Maximum Likelihood method based on the Jukes-Cantor model. Number on the nodes represent bootstrap percentages (1000 replicates). Accession numbers for sequences from GenBank are shown.

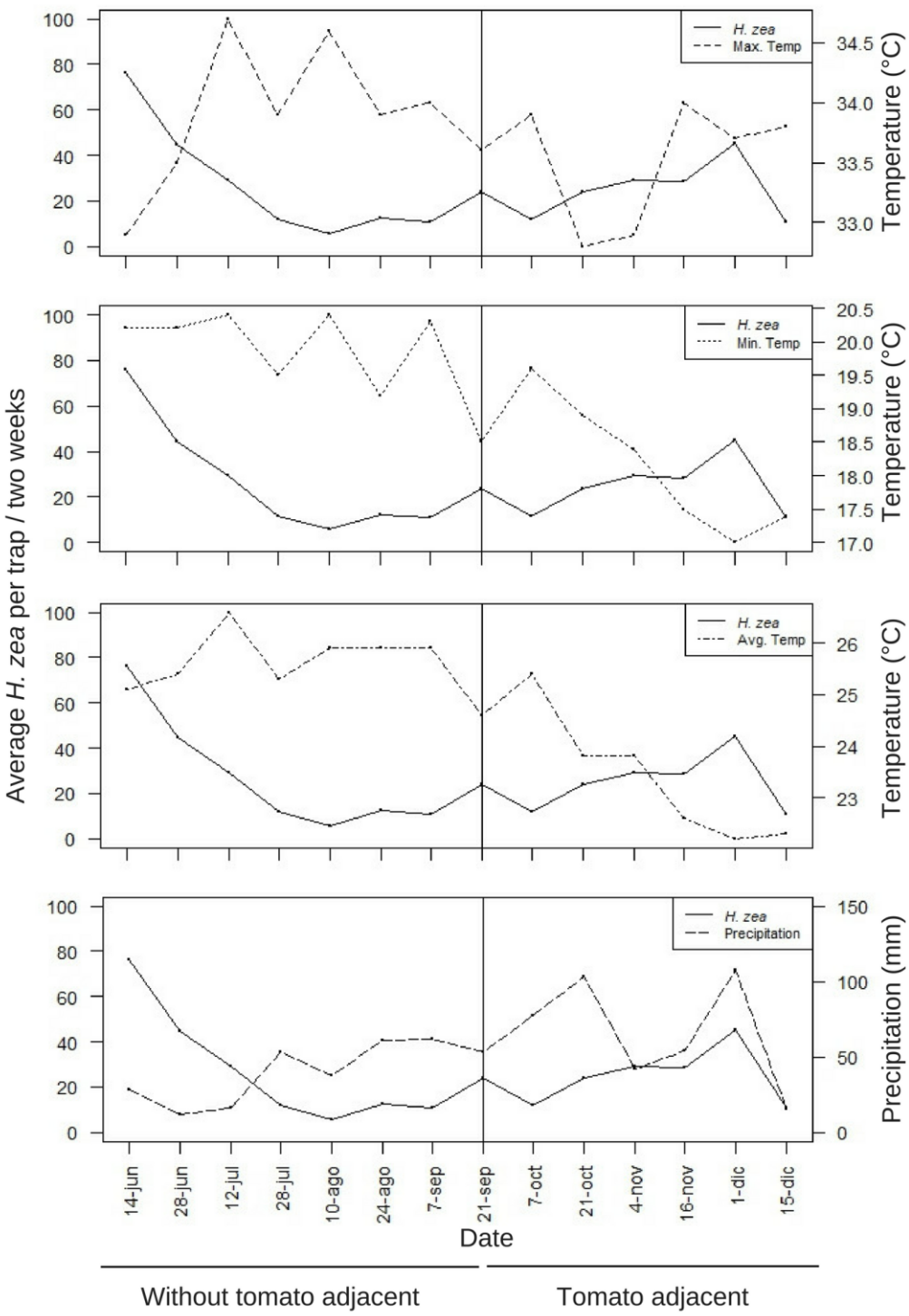

Figure 11: *Helicoverpa zea* captured on traps at bell pepper farm and weather variables for regression analysis. June to December 2016, Santa Isabel, PR. Graphs are divided in the periods that the bell pepper farm had or not tomato adjacent to the grange.

6. Appendices

Appendix 1: Traps location, crops near to *Helicoverpa zea* detections, date, and counts of *H. zea* per trap. February 2016 to January 2017.

Trap	Locality	Crop	Latitude	Longitude	Date of trap installation	Date of trap removement	*H. zea*/ trap
1	Isabela	Soybean	18.47076	-67.04997	Feb 3, 2016	Jun27, 2016	75
2	Isabela	Soybean	18.46322	-67.0537	Jun27, 2016	Aug11, 2016	1
3	Isabela	Soybean	18.4711	-67.04575	Aug11, 2016	Jan11, 2017	3
4	Isabela	Soybean	18.46615	-67.05408	Aug31, 2016	Nov10, 2016	9
5	Isabela	Soybean	18.39112	-66.05407	Sep8, 2016	Oct5, 2016	0
6	Isabela	Soybean	18.46347	-67.05271	Oct5, 2016	Nov10, 2016	0
7	Isabela	Soybean	18.47115	-67.04572	Oct5, 2016	Nov22, 2016	1
8	Isabela	Soybean	18.4715	-67.04487	Nov22, 2016	Jan11, 2017	2
9	Isabela	Corn	18.46305	-67.05431	Feb3, 2016	Mar16, 2016	13
10	Isabela	Corn	18.46396	-67.05505	Nov22, 2016	Jan11, 2017	1
11	Isabela	Corn	18.46345	-67.05486	Nov22, 2016	Jan11, 2017	2
12	Isabela	Pigeon pea	18.46378	-67.05574	Feb3, 2016	Mar9, 2016	22
13	Isabela	Crotalaria	18.46653	-67.04546	Mar30, 2016	Jun10, 2016	74
14	Isabela	Sorghum	18.47141	-67.14789	Jun27, 2016	Oct5, 2016	3
15	Isabela	Bean	18.47093	-67.04815	Aug11, 2016	Oct25, 2016	16
16	Mayagüez	Chili pepper	18.21025	-67.14218	Feb4, 2016	Jan11, 2017	0
17	Mayagüez	Chili pepper	18.21064	-67.1443	Feb24, 2016	Aug26, 2016	0
18	Mayagüez	Chili pepper	18.21764	-67.14789	Jun14, 2016	Oct25, 2016	0
19	Mayagüez	Pigeon pea	18.18091	-67.14435	Feb24, 2016	Jun14, 2016	0
20	Mayagüez	Corn	18.21997	-67.1443	Feb4, 2016	Jun14, 2016	1
21	Mayagüez	Corn	18.21991	-67.14683	Sep8, 2016	Jan11, 2017	2
22	Mayagüez	Corn	18.21064	-67.1443	Oct25, 2016	Jan11, 2017	0
23	Guanica	Bell pepper	17.97986	-66.90089	Feb15, 2016	Apr4, 2016	79
24	Guanica	Bell pepper	18.07454	-66.96381	Feb22, 2016	May2, 2016	52
25	Guanica	Tomato	17.97760	-66.90021	Feb15, 2016	Apr4, 2016	29
26	Guanica	Tomato	17.98615	-66.90225	Apr18, 2016	Jun14, 2016	124
27	Guanica	Tomato	17.98618	-66.90318	Nov16, 2016	Jan13, 2017	8
28	Guanica	Chili pepper	17.98633	-66.90435	Apr18, 2016	Aug24, 2016	74
29	Guanica	Chili pepper	17.98781	-66.90378	Apr18, 2016	Aug24, 2016	42
30	Guanica	Chili pepper	18.00745	-66.88863	May16, 2016	Jul26, 2016	43
31	Guanica	Chili pepper	18.00718	-66.88905	Dec15, 2016	Jan13, 2017	13
32	Guanica	Okra	17.98766	-66.90279	Aug10, 2016	Jan13, 2017	37
33	Guanica	Okra	17.98621	-66.90302	Aug24, 2016	Jan13, 2017	66
34	Guanica	Cotton	17.99513	-66.96078	Mar21, 2016	May31, 2016	39
35	Guanica	Cotton	17.9976	-66.9594	May31, 2016	Dec15, 2016	42
36	Guanica	Cotton	17.99754	-66.95872	Oct7, 2016	Dec15, 2016	13

Continuation Appendix 1

Trap	Locality	Crop	Latitude	Longitude	Date of trap installation	Date of trap removement	*H. zea*/ trap
37	Guanica	Cotton	18.00055	-66.95821	Dec15, 2016	Jan13, 2017	1
38	Guanica	Soybean	17.99534	-66.96012	Mar21, 2016	May31, 2016	37
39	Guanica	Soybean	18.00068	-66.95895	May31, 2016	Aug10, 2016	1
40	Guanica	Soybean	17.99646	-66.95614	Aug10, 2016	Oct7, 2016	14
41	Guanica	Soybean	17.99468	-66.95896	Dec15, 2016	Jan13, 2017	0
42	Guanica	Sunflower	18.00935	-66.89254	Feb15, 2016	Jan13, 2017	135
43	Guanica	Sunflower	18.00923	-66.89365	May2, 2016	Jun14, 2016	9
44	Guanica	Sunflower	18.01208	-66.89384	May16, 2016	Jun14, 2016	1
45	Guanica	Sunflower	18.01036	-66.89438	Jun14, 2016	Jun28, 2016	1
46	Guanica	Sunflower	18.00935	-66.89368	Jun28, 2016	Aug24, 2016	13
47	Guanica	Sunflower	18.01197	-66.89331	Jul12, 2016	Aug24, 2016	3
48	Guanica	Sunflower	18.01066	-66.89372	Jul12, 2016	Aug24, 2016	7
49	Guanica	Sunflower	18.00912	-66.89184	Aug24, 2016	Oct7, 2016	14
50	Guanica	Sunflower	18.00907	-66.89039	Aug24, 2016	Oct7, 2016	5
51	Guanica	Sunflower	18.00834	-66.89039	Aug24, 2016	Nov4, 2016	10
52	Guanica	Sunflower	18.00766	-66.89040	Oct7, 2016	Nov4, 2016	1
53	Guanica	Sunflower	18.07840	-66.89093	Oct7, 2016	Nov4, 2016	2
54	Guanica	Sunflower	18.00930	-66.89240	Nov4, 2016	Jan13, 2017	8
55	Guanica	Sunflower	18.00979	-66.89256	Nov4, 2016	Jan13, 2017	0
56	Guanica	Sunflower	18.01505	-66.89241	Nov4, 2016	Jan13, 2017	6
57	Guanica	Sunflower	18.01125	-66.89316	Dec15, 2016	Jan13, 2017	1
58	Guanica	Pumpkin	18.00721	-66.89000	Aug24, 2016	Dec15, 2016	7
59	Guanica	Pumpkin	17.98607	-66.90741	Jul26, 2016	Nov16, 2016	16
60	Guanica	Pumpkin	17.98636	-66.90577	Aug24, 2016	Nov16, 2016	17
61	Guanica	Pumpkin	17.98631	-66.90435	Nov16, 2016	Jan13, 2017	2
62	Santa Isabel	Tomato	17.98297	-66.41855	Feb15, 2016	May9, 2016	859
63	Santa Isabel	Tomato	17.99873	-66.41795	Feb15, 2016	May9, 2016	837
64	Santa Isabel	Tomato	17.99769	-66.42598	Apr18, 2016	May31, 2016	199
65	Santa Isabel	Tomato	17.99756	-66.42691	Apr18, 2016	May31, 2016	166
66	Santa Isabel	Bell pepper	17.98287	-66.43085	May2, 2016	May9, 2016	44
67	Santa Isabel	Bell pepper	17.98493	-66.42953	May16, 2016	Jan13, 2017	735
68	Santa Isabel	Bell pepper	17.98547	-66.42914	May16, 2016	Jun14, 2016	151
69	Santa Isabel	Bell pepper	17.98369	-66.42665	May16, 2016	Jul28, 2016	187
70	Santa Isabel	Bell pepper	17.98645	-66.42850	Jun14, 2016	Jan13, 2017	189
71	Santa Isabel	Bell pepper	17.98494	-66.42641	Jun14, 2016	Jan13, 2017	253
72	Santa Isabel	Bell pepper	17.98717	-66.42809	Jul28, 2016	Jan13, 2017	201
73	Lajas	Corn	18.03095	-67.07239	Mar7, 2016	Apr18, 2016	0
74	Lajas	Corn	18.03219	-67.07036	Mar21, 2016	Apr18, 2016	1

Continuation Appendix 1

Trap	Locality	Crop	Latitude	Longitude	Date of trap installation	Date of trap removement	*H. zea*/ trap
75	Lajas	Corn	18.03189	-67.07178	Apr18, 2016	May31, 2016	3
76	Lajas	Corn	18.39130	-66.05697	May31, 2016	Jul26, 2016	3
77	Lajas	Corn	18.02588	-67.07491	May31, 2016	Jul26, 2016	2
78	Lajas	Chili pepper	18.03091	-67.07386	Jul12, 2016	Aug26, 2016	4
79	Lajas	Chili pepper	18.03030	-67.07406	Jul12, 2016	Jan13, 2017	8
80	Lajas	Rice	18.02981	-67.07657	Aug10, 2016	Jan13, 2017	0
81	Lajas	Pumpkin	18.03091	-67.07386	Aug26, 2016	Oct5, 2016	0
82	Guayama	Soybean	17.98781	-66.21546	Jul26, 2016	Nov16, 2016	12
83	Guayama	Soybean	17.98719	-66.21555	Aug10, 2016	Nov16, 2016	5
84	Juana Diaz	Bean	18.02813	-66.52991	Feb22, 2016	Apr18, 2016	25
85	Juana Diaz	Bean	18.03151	-66.52896	Jul28, 2016	Nov4, 2016	4
86	Gurabo	Plantain	18.25630	-65.98750	Apr7, 2016	May6, 2016	0
87	Gurabo	Plantain	18.25510	-65.98700	Apr7, 2016	May6, 2016	0
88	Gurabo	Plantain	18.25312	-65.99129	Apr7, 2016	May6, 2016	0
89	Gurabo	Pigeon pea	18.25262	-65.98969	Apr7, 2016	May6, 2016	0
90	Gurabo	Chili pepper	18.25160	-65.98969	Apr7, 2016	May6, 2016	0
91	Gurabo	Chili pepper	18.25518	-65.99218	May6, 2016	Dec14, 2016	0
92	Gurabo	Chili pepper	18.25571	-65.99151	May6, 2016	Dec14, 2016	0
93	Gurabo	Chili pepper	18.25268	-65.98972	May6, 2016	Dec14, 2016	0
94	Gurabo	Soybean	18.25125	-65.9901	May6, 2016	Dec14, 2016	0
95	San Juan	Royal palm	18.39043	-66.05346	Apr8, 2016	Dec14, 2016	0
96	San Juan	Chili pepper	18.38829	-66.05653	Apr8, 2016	Dec14, 2016	0
97	San Juan	Chili pepper	18.39455	-66.06505	Apr8, 2016	Dec14, 2016	0
98	San Juan	Weeds	18.39054	-66.05305	Apr8, 2016	Dec14, 2016	0
99	Añasco	Corn	18.27339	-67.15700	Feb3, 2016	Feb24, 2016	16
100	Añasco	Chili pepper	18.27470	-67.15669	Feb3, 2016	Feb24, 2016	11
101	Aguadilla	Pigeon pea	18.44705	-67.12185	Feb3, 2016	Feb24, 2016	4
102	Sabana Grande	Pigeon pea	18.08432	-66.94881	Feb15, 2016	Feb22, 2016	2
103	Villalba	Pigeon pea	18.13263	-66.49481	Sep27, 2016	Jan18, 2017	0
104	Villalba	Pigeon pea	18.13238	-66.49500	Sep27, 2016	Jan18, 2017	0
105	Villalba	Pigeon pea	18.13247	-66.49511	Sep27, 2016	Jan18, 2017	0
106	Villalba	Pigeon pea	18.13325	-66.49448	Sep27, 2016	Jan18, 2017	0
107	Villalba	Pigeon pea	18.14778	-66.4508	Oct28, 2016	Jan18, 2017	0
108	Jayuya	Pigeon pea	18.19428	-66.53786	Sep27, 2016	Jan18, 2017	0
109	Jayuya	Pigeon pea	18.19469	-66.53776	Sep27, 2016	Jan18, 2017	0
110	Jayuya	Pigeon pea	18.19419	-66.53747	Sep27, 2016	Jan18, 2017	0
111	Jayuya	Pigeon pea	18.21014	-66.55663	Sep27, 2016	Jan18, 2017	0
112	Jayuya	Pigeon pea	18.21059	-66.55596	Sep27, 2016	Jan18, 2017	0

Appendix 2: *Helicoverpa zea* captures in yellow bucket Unitraps in bell pepper farm. Data for regression analysis with weather variables. Santa Isabel, PR.

Date	Trap 1	Trap 2	Trap 3	Trap 4
Jun 14	95	64	69	76*
Jun 28	65	43	22	48
Jul 12	48	28	16	25
Jul 28	11	4	18	15
Aug 10	8	6	3	6*
Aug 24	27	13	2	7
Sep 7	16	5	1	22
Sep 21	50	10	17	19
Oct 7	24	1	8	14
Oct 21	44	10	16	26
Nov 4	54	14	29	20
Nov 16	76	17	13	8
Dec 1	84	24	30	41
Dec 15	21	13	7	3

***** Trap fell this date, data shown average of the other traps.

Appendix 3: Weather data from Agricultural Experimental substation of the University of Puerto Rico at Juana Diaz, used for regression analysis in bell pepper farm, Santa Isabel, PR.

Date	Maximum temperature (°C) [1]	Minimum temperature (°C)	Average temperature (°C)	Precipitation (mm)[2]
Jun 14	32.9	20.2	25.1	28.19
Jun 28	33.5	20.2	25.4	11.68
Jul 12	34.7	20.4	26.6	16.26
Jul 28	33.9	19.5	25.3	53.12
Aug 10	34.6	20.4	25.9	38.10
Aug 24	33.9	19.2	25.9	60.96
Sep 7	34.0	20.3	25.9	62.23
Sep 21	33.6	18.5	24.6	53.59
Oct 7	33.9	19.6	25.4	77.63
Oct 21	32.8	18.9	23.8	103.63
Nov 4	32.9	18.4	23.8	42.42
Nov 16	34.0	17.5	22.6	54.22
Dec 1	33.7	17.0	22.2	107.16
Dec 15	33.8	17.4	22.3	15.49

1: Temperatures shown are averages since the last sampling until date of monitoring.

2: Precipitation shown is accumulated since the last sampling until date of monitoring.

CHAPTER III: Morphometrics of *Helicoverpa armigera* (Hübner), *Helicoverpa zea* (Boddie), and Their Hybrids (Lepidoptera: Noctuidae)

Abstract

Hybrids between males *H. armigera* and females *H. zea* were obtained at Center for Excellence in Quarantine and Invasive Species laboratory, San Juan Puerto Rico. *Helicoverpa zea* was originated from Puerto Rico, while *H. armigera* was from Brazil. The identification of insects from colonies and hybrids was performed with specific primers of the ITS1 region. In the cross between males *H. armigera* x females *H. zea,* 21.4 % of the females copulated, and 11.6 % of the eggs were fertile. In the cross between males *H. zea* x females *H. armigera,* no fertile egg was produced. The pupal weight of F1 hybrids was similar to *H. zea,* and *H. armigera* had the lowest pupal weight. For morphological comparison, the following quantitative variables were analyzed: number of lobes, number of cornuti, length of valves (mm), length of aedeagus (mm), and depth in the posterior excavation in the eighth sternite (mm). Qualitative variables of the eighth abdominal sternite were evaluated: shape of distal apices, proximal margin, and distal margin. *Helicoverpa armigera* had one lobe while F1 hybrids and *H. zea* showed three lobes. In the remaining morphological variables were observed overlaps between *H. armigera* and *H. zea.* Statistical analysis for the number of cornuti, length of valves, length of aedeagus, and depth in the posterior excavation in the eighth sternite, showed that *H. zea* had the biggest structures or more cornuti, than hybrids with smaller size of structures or less cornuti than *H. zea,* and *H. armigera* with smallest structure sizes and reduced number of cornuti. During the evaluation of qualitative variables, using the shape of the distal apices and proximal margin in the eighth sternite was difficult to infer if hybrids were closer to *H. zea* or *H. armigera* due to the ambiguity of shapes; in the shape of the distal margin, hybrids were closer to *H. armigera*. In the principal components analysis, with qualitative variables, F1 hybrids were more relative to *H. zea* than *H. armigera.* Considering the analyzed characters, the use of only morphology is very difficult to identify one *Helicoverpa* as a hybrid.

1. Introduction

The moths *Helicoverpa armigera* (Hübner) and *Helicoverpa zea* (Boddie) are serious pests in many crops (Hardwick, 1965). *Helicoverpa armigera* could affect more than 180 plants, while *H. zea* attack 123 plants, both insects affect crops and weeds (Cunningham &

Zalucki, 2014; Kogan, et al., 1989). Both Lepidoptera have developed resistance to some insecticides, however *H. armigera* show resistance to more insecticides and resistance factors are higher than *H. zea* (Kranthi, et al, 2005; Mahon, et al., 2007; Pérez et al., 2000; Pietrantonio et al., 2007; Ali, et al., 2006). *Helicoverpa zea* is distributed in the New World (Hardwick, 1965). *Helicoverpa armigera* was distributed in the Old World, since 2013 this pest was reported in America, the first detection was in Brazil (Czepak & Albernaz, 2013), then was detected in Argentina, Uruguay, Paraguay, Bolivia, Dominican Republic, Peru, and Puerto Rico (Arnemann et al., 2016; Gilligan et al., 2015; Kriticos et al., 2015; Murúa et al., 2014; USDA-APHIS-PPQ, 2014). *Helicoverpa armigera* and *H. zea* are species close related, their external morphology is identical, and then these moths have been considered conspecific species (Hardwick, 1965; Pogue, 2004). Species delimitation of these insects is given by morphological differences in genitalia of males and females (Hardwick, 1965), differences in their genome (Anderson et al., 2016; Cho et al., 2008), and range of hosts: *H. armigera* attack more hosts than *H. zea*, however some of these hosts are the same for both insects (Cunningham & Zalucki, 2014). Behere et al. (2007) suggest that *H. zea* derived from *H. armigera* around 1.5 million years ago. Pearce et al. (2017) report that in this process of derivation, *H. zea* lost some detoxification genes, and certain genes that confer resistance to insecticides.

In animals, 10 % of the species could crossbreed with distinct species; in the genus *Heliconius* (Lepidoptera: Nymphalidae) 34.8 % of the species could hybridize (Mallet, 2005). In some cases hybridization could lead to speciation, this has been suggested for birds *Geospiza* (Lamichhaney et al., 2017), *Lepidothrix* (Barrera-Guzmán et al., 2017), butterfly *Heliconius* (Mavárez et al., 2006). Hybrids usually have less survival and are less fertile than their parents (Mallet, 2005), in some cases one sex is sterile or absent (Haldane, 1922). But in other cases, the hybrids have advantages over their parentals, such as more survival and adaptation to new environments (Grant & Grant, 1992; Gross & Rieseberg, 2005; Lewontin & Birch, 1966). Hybridization between *H. armigera* and *H. zea*, under laboratory conditions, has been reported by Hardwick (1965), Laster & Sheng (1995), and Laster & Hardee (1995). Lepidoptera hybrids were founded in nature: *Heliconius* (Nymphalidae) and *Papilio* (Papilionidae) (Aubert et al., 1997; Mallet, 2005). In Brazil has been found in the field possible hybrids of *H. armigera* and *H. zea* (Anderson et al., 2016, 2018; Leite et al., 2016,

2017; Lopes et al., 2017). Therefore, hybridization between *H. armigera* and *H. zea* in nature is a possible event, in this case, is a possibility that hybrids share characteristics of both parentals and have advantages in the environment (Mallet, 2018).

Helicoverpa armigera is an invasive species in America, and its resistance to insecticides is a point of concern (Kranthi et al., 2005; Kriticos et al., 2015). Surveys for detection of *Helicoverpa* are usually performed using morphology of the male genitalia (Brambila, 2009). The objective of the research was to compare the morphology of the male genitalia of hybrids with the parental species *H. armigera* and *H. zea.*

2. Materials and Methods

2.1. Colonies of *Helicoverpa*

The initial colony of *H. zea* was obtained from larvae collected in Isabela, Puerto Rico, in pigeon pea fields on November 11, 2015. During the generation F9, a reintroduction of insects was done, from larvae collected in Isabela in corn on November 22, 2016. The colony of *H. armigera* was obtained from five larvae and 30 pupae from Brazil, these insects were introduced to quarantine facilities of the Center of Excellence in Quarantine & Invasive Species on February 4, 2017, under Puerto Rico Department of Agriculture Permit number OV-1617-03 and USDA-APHIS Permit number P526P-15-04600 to Dr. José Carlos Verle Rodrigues. It was not possible to work with a colony of *H. armigera* from Puerto Rico because the populations in the island are still low, see results Chapter II.

The insects were identified with the morphology of male genitalia following the methods of Pogue (2004) and Brambila (2009). For molecular identification DNA extraction was done with methods described in chapter II, for the extraction were used thorax, head, or larval exuvia of the insects. PCR assays were done with specific primers for ITS1 region: 3373Ha_Hz_ITS1-F (common forward), 3374Ha_ITS1-R *(H. armigera* specific reverse), and 3377Hz_ITS1-R *(H. zea* specific reverse). For each reaction of 25 µl was used a mixture of 12,5 µl of PCR Master Mix Promega 2X (Promega, Cat. Number: M7505, USA 1 µl of primer 3373Ha_Hz_ITS1-F (10 µM), 1 µl of primer 3374Ha_ITS1-R (10 µM), 0.5 µl of primer 3377Hz_ITS1-R (10 µM), and 2-4 µl of DNA template, the volume of water was adjusted to 25 µl. The PCR program was 45s minutes at 95°C, 35 cycles of 15s at 95°C, 10s at 60°C, and 30s at 72°C, and a final extension at 72°C for five minutes. The amplicons were

checked by 1.2 % agarose gel electrophoresis in buffer TBE (Tris-borate-EDTA). Using these primers is possible to identify *H. armigera* and *H. zea* without sequencing; *H. armigera* has amplicons of ≈147 bp, while *H. zea* has amplicons of ≈343 bp (Perera et al., 2015).

2.2. Insects rearing

The insects were reared in the quarantine facilities of the Center of Excellence in Quarantine and Invasive Species (CEQIS), San Juan Puerto Rico. Larvae, male pupae, adults, and eggs were maintained in containing rooms at 25±2 °C, 57±9 % relative humidity, photoperiod of 15 hours of light and 9 hours of dark (15: 9 LD). Female pupae were placed in incubators at 22.7 ± 1.6 °C, 82 ±4 % relative humidity, photoperiod of 15:9 LD, females were placed at less temperature to synchronize the emergence of adults with males (Armes et al, 1992; Colvin & Cooter, 1994). The larvae were fed with Gypsy Moth Diet (Frontier Agricultural Sciences, Product # F9630B, Newark DE): 140.2 g of dry mix, 20 g of fats and sugars, 1.6 g of vitamin mix, 0.8 g of aureomycin, 1000 ml of distilled water, with the addition of 12 ml of formaldehyde 1%, and 2.5 g of FABCO mold inhibitor (Frontier Agricultural Sciences, Product # F0018, Newark DE); the agar was dissolved, when the temperature was ~50°C the rest of the reagents were added. Each larva was maintained in transparent plastic cups of 30 ml containing diet. The pupae were maintained in the same cups. Emerged adults and pupae near to emerge were placed in white plastic buckets of 18.9 l, the upper part of the buckets was covered with cheesecloth (DeRoyal, BIDF2012380-BX, Tennessee) for oviposition of the females. Inside each bucket a Petri dish with autoclaved washed sand was placed to increase the relative humidity, each bucket had a potted tomato plant inside. The adults received the following diet recipe modified from Grzywacz et al. (2002): 500 ml of distilled water, 50 ml of honey, 10 ml of solution 28 % of Vanderzant vitamin mixture (Sigma, V1007, USA), 1 g of methyl-4-hydroxybenzoate (Sigma, H3647, USA), and 1 ml of ethanol 95 %; methyl-4-hydroxybenzoate was dissolved in the ethanol 95 %, then all the ingredients were mixed in the water. The cheesecloth with the laid eggs was placed in Ziploc® bags of 3.8 l with fine strips of larval diet. Once larvae emerged, they were transferred to the cups with diet. The cups were placed in plastic trays for 30 cups.

2.3. Hybridization assays

For hybridization crosses, 15 male pupae and 15 female pupae were placed together in the buckets for the emergence of adults. The following variables were taken: percentage

of copulated females, percentage of fertile eggs, and pupal weight. The pupal weight was analyzed with ANOVA using a randomized complete block design, each tray for 30 insects was considered a block. Prior the analysis Shapiro test and Levene test were done to verify normality and homogeneity of the data. The statistical analysis was done with R software (version 3.3.2) with the package agricolae (Mendiburu, 2017).

DNA was extracted from larval or pupal exuvia; the extraction was done with methods described in chapter II. Real Time PCR assays were done with primers for Internal transcribed spacer (ITS)1 region specific for *H. armigera* and *H. zea* (Perera et al., 2015), these assays were done with methods described in chapter II. Using these specific primers, *H. zea* samples show a peak of fluorescence at 87.5 to 88.5 °C in the melt analysis for Real Time PCR, *H. armigera* show a peak of fluorescence at 82.8 to 85.5 °C with the equipment used, and samples of F1 hybrids between *H. armigera* and *H. zea* should have two peaks of fluorescence at same temperatures than *H. armigera* and *H. zea*, similar than predicted by Perera et al. (2015).

2.4. Morphological comparison of *H. armigera, H. zea,* and their hybrids

Morphology of male F1 hybrids (♂ *H. armigera* x ♀ *H. zea)* (n=30) were compared with *H. armigera* (n=30), 21 were from Brazil, reared in the laboratory at the Center of Excellence in Quarantine and Invasive Species, five from Spain reared in laboratory and provided by Dra. Hannah Nadel (USDA, APHIS Otis Lab. Buzzards Bay, MA), and four from Puerto Rico captured in yellow bucket Unitraps; and *H. zea* (n=30) from Puerto Rico, 21 from Isabela and reared in laboratory at the Center of Excellence in Quarantine and Invasive Species, and nine captured in traps: four from Santa Isabel, and five from Guanica. Molecular identification of *Helicoverpa* from Spain and captured insects in bucket traps was done with Real Time PCR using primers for ITS1 region with methods described in chapter II.

Male genitalia of the insects was extracted using the methods described in chapter II. The following quantitative morphological characters were checked: number of lobes in the vesica (Figure 4), number of cornuti in the aedeagus, length of aedeagus (mm) (Figure 5), length of valves (mm) (Figure 6), depth of concavity in the eighth abdominal sternite (mm) (Figure 7). Qualitative variables were checked: shape of distal apices in the eighth abdominal sternite (Figure 8), shape of proximal margin in the eighth abdominal sternite (Figure 9),

these two last variables were qualified according to Pogue (2004); additionally was qualified the shape of distal margin in the eighth abdominal sternite in this manner: U-shape as in Figure 10D, or V-shape with rounded apex as in Figures 10A-C. The qualitative variables of shape were qualified by five volunteer persons to get a consensus of each sample, these persons qualified the photographs of the structures, the descriptions and photographs of distal apices and proximal margin given by Pogue (2004) were provided to these persons, if one sample was qualified in a different manner for two persons that sample was considered as ambiguous shape. The photographs and measurements were taken with a stereomicroscope Leica M80 using a camera Leica DMC 2900.

The variables number of cornuti, length of aedeagus, length of valves, and depth of concavity in the eighth sternite were analyzed with ANOVA using a completely random design; the variable length of valves was transformed (x^3) to get normal data. Prior the analysis Shapiro test and Levene test were done to verify normality and homogeneity of the data. The statistical analysis was done with R software with the package agricolae (Mendiburu, 2017).

To determine if the morphology of male genitalia of F1 hybrids is more related to *H. zea* or *H. armigera*, principal component analysis (PCA) was done with software Past, version 3.18 (Hammer et al., 2001), the following variables were used: number of lobes, number of cornuti, length of aedeagus (mm), length of valves (mm), and depth of concavity in the eighth sternite (mm). Prior the analysis each data was transformed dividing for the standard deviation of each variable, in the software was selected the Matrix Correlation because the variables were taken in different units (Hammer et al., 2001), bootstrap of 10,000 was used in the analysis.

3. Results and discussion

3.1. Identification of colonies *H. armigera* and *H. zea*

Insects from *H. zea* colony showed three lobes in the vesica, and *H. armigera* colony one lobe in the vesica, this character is essential to distinguish these species (Brambila, 2009). In the PCR assays with specific primers for ITS1 region, insects from *H. zea* colony showed amplicons of ~ 343 bp (Figure 1), while *H. armigera* colony showed amplicons of ~ 147 bp (Figure 2), these sizes of amplicons corroborates identifications of both colonies according to Perera et al. (2015).

3.2. Hybridization assays

The results of these experiments are resumed in Table 1. *Helicoverpa armigera* and *H. zea* showed 45 and 50 % of copulated females respectively; Laster & Sheng (1995) report a similar percentage of copulated females for these species. In the current research *H. armigera* and *H. zea* had 60 and 61.8 % of fertile eggs respectively. Laster & Sheng (1995) report 46.4 % of fertile eggs for *H. armigera,* and 70 % for *H. zea.*

Only in the cross ♂ *H. armigera* x ♀ *H. zea* were obtained F1 generation. In the Real Time with ITS1 primers the F1 hybrids showed two peaks of fluorescence in the melt analysis at similar temperatures than *H. armigera* and *H. zea* (Figure 3), this confirmed that these insects are hybrids as predicted by Perera et al. (2015). In the bucket for this cross emerged 15 males and 14 females, three females were copulated (21.4 %). Laster & Sheng (1995) report 29.6 % of copulated females for this cross, similar to the observed in the current research. Hardwick (1965) attempted this cross twice but he could not obtain fertile eggs, however, he observed copulated females. In the current research, this cross showed 11.53 % of fertile eggs from a total of 1,959 eggs, nevertheless in the total number of eggs are included laid by non-copulated females, and by mated but not reproducing females. Laster & Sheng (1995) report 31 % of fertile eggs for this cross. In the current experiment were obtained 226 larvae of F1 hybrids, 31 males and 29 females reached the adult stage. Laster & Sheng (1995), and Laster & Hardee (1995) did not find sex distortion ratio either in the F1 hybrids in the crosses between *H. armigera* and *H. zea.* Zhao et al. (2005), Tang (2005), and Wang & Dong (2001) report in the cross ♂ *Helicoverpa assulta* x ♀ *H. armigera*, that F1 hybrids had presence only of males. In the current research, the pupal weight of F1 hybrids (♂ *H. armigera* x ♀ *H. zea)* was statistically equal to *H. zea*, and *H. armigera* pupal weight was less than *H. zea* and hybrids (*P-value* = 0.0006, Tukey test α=0.05). Laster & Sheng (1995) report that *H. zea* had higher pupal weight than F1 hybrids (♂ *H. armigera* x ♀ *H. zea*), and F1 hybrids had higher pupal weight than *H. armigera.* In the current investigation, any F1 hybrid (♂ *H. armigera* x ♀ *H. zea)* showed abnormal genitalia or sterility; Laster & Hardee (1995) and Laster & Sheng (1995) did not find either sterile males or abnormal genitalia in hybrids or in the backcrosses between *H. armigera* and *H. zea.* Aubert (2017) did not find sterility in F1 hybrids or in the backcrosses in hybridization assays between *Papilio hospiton* (Géné) and *P. macahon* (L.) (Lepidoptera: Papilionidae). Zhao et al. (2005) founded

malformations in the genitalia in some males of F1 hybrids in the cross ♂ *H. assulta* x ♀ *H. armigera*, but in the cross ♂ *H. armigera* x ♀ *H. assulta* the F1 hybrids did not show deformations.

In the cross ♂ *H. zea* x ♀ *H. armigera*, emerged ten males and 14 females, two females copulated (14.2 %), females laid 21 eggs, but any egg was fertile. In this cross were observed pairs locked during the copula (pairs that could not separate), this is an event that affects the mating in *Helicoverpa* (Hardwick, 1965). Laster & Sheng report 4.5 % of copulated females for this cross, and 26.7 % of copulated females were reproducing. Laster & Hardee (1995) did this cross with two females, with both females copulating and laying fertile eggs. Hardwick (1965) report one female laying fertile eggs of 30 females attempted for this cross.

Degrugillier & Newman (1993) report F1 hybrids for the cross ♂ *H. zea* x ♀ *H. assulta*. Hardwick (1965) tried to hybridize *H. armigera* with *H. punctigera*, the author founded copulated females, but the eggs were not fertile.

3.3. Overlaps in the morphology of *H. armigera* and *H. zea*

Quantitative variables for these species are resumed in Table 2 and Figure 11. All *H. zea* had three lobes in the vesica while *H. armigera* had one lobe (Figure 4A-B); the number of lobes is an essential character to distinguish these species (Brambila, 2009), however in this research were observed overlaps in the morphology for the other variables analyzed. The statistical analysis shows that *H. zea* had bigger structures or more cornuti than *H. armigera* (*P-value*<0.001, Tukey test α=0.05), but minimum and maximum values of the variables analyzed showed overlaps. *H. zea* had 15-21 cornuti, and *H. armigera* 9-15 cornuti, one *H. zea* from Puerto Rico captured in a bucket trap had 15 cornuti, and one *H. armigera* from Spain had 15 cornuti too. Balbi et al. (2017) report one *H. zea* with 15 cornuti too. Pogue (2004) indicates that *H. zea* has more cornuti than *H. armigera*, in this research, it was founded the same situation, but in case of one *Helicoverpa* has 15 cornuti other morphological characters should be checked for a correct identification. Length of valves in *H. zea* was between 4.15 to 5.09 mm, while in *H. armigera* 3.50 to 4.74 mm. *Helicoverpa zea* with the smallest valves (4.15 mm) was from Puerto Rico, Laboratory colony; and *H. armigera* with the biggest valves (4.74 mm) was from Spain. Pogue (2004) reports similar ranks for this variable: *H. zea* with length of valves of 4.65 to 5.40 mm, while *H. armigera*

with length of valves of 4.10 to 4.85 mm. *Helicoverpa zea* had length of aedeagus of 4.55 to 5.83 mm, while *H. armigera* length of aedeagus of 3.29 to 4.84 mm, *H. zea* with the smallest aedeagus (4.55 mm) was from Puerto Rico captured in the field; and *H. armigera* with the biggest aedeagus (4.84 mm) was from Spain. *Helicoverpa zea* had a depth in the posterior excavation of eighth sternite in a rank of 0.42 to 0.76 mm, while *H. armigera* a rank of 0.29 to 0.52 mm, *H. zea* with the minimum value for this variable (0.42 mm) and *H. armigera* with the maximum value for this character (0.52 mm) were from Puerto Rico captured in bucket traps. Complete data of quantitative variables is in Appendix 1.

Qualitative variables of these species are resumed in Table 3. Pogue (2004) reports that *H. zea* has the distal apices in the eighth sternite more rounded, and *H. armigera* have distal apices more pointed. In this research 89.3 % of *H. zea* had rounded apices as in Figure 8E; 3.6 % of *H. zea* had pointed distal apices (Figure 8D), this corresponds to one sample from Puerto Rico captured in a bucket trap; and 7.1% of *H. zea* had an ambiguous shape of distal apices (Figure 8F), this represents two samples from Puerto Rico captured in bucket traps. In *H. armigera* 63 % of samples had pointed distal apices (F igure 8A), 18.5 % of *H. armigera* showed rounded distal apices (Figure 8B), and 18.5 % *H. armigera* had an ambiguous shape (Figure 8C), samples with rounded and ambiguous shape for this character were from Brazil. Majority of *H. zea* and *H. armigera* had the shape of distal apices as descriptions suggested by Pogue (2004) for these species. Pogue (2004) indicates that *H. zea* have the proximal margin in eighth sternite as U-shape, while *H. armigera* have as V-shape with apex flattened. In this investigation 86.2 % of samples had U-shape in the proximal margin as in Figure 9D; any *H. zea* had V-shape with apex flattened, and 13.8 % of *H. zea* had ambiguous shape (Figures 9E-F), this corresponds to four samples from Puerto Rico, three from laboratory colony, and one captured in a bucket trap. In *H. armigera* 25.9 % of samples had a proximal margin as V-shape with apex flattened (Figure 9B), 44.4 % of *H. armigera* had proximal margin with U-shape, as in Figure 9A, this corresponds to 12 samples, three from Spain, eight from Brazil, and one from Puerto Rico, and 29.6% of *H. armigera* had ambiguous shape of proximal margin (Figure 9C), this corresponds to eight samples, seven from Brazil, and one from Spain. Majority of *H. zea* had the shape of proximal margin as described by Pogue (2004), but in *H. armigera* the shape of proximal margin was very variable. In addition, was qualified the shape of distal margin in the eighth sternite in this

manner: U-shape as in Figure 10D, or V-shape with rounded apex as in Figures 10A-C. *Helicoverpa zea* had 86.2 % of the samples as U-shape in the distal margin (Figure 10D); 6.9% of *H. zea* had V-shape with apex rounded (Figure 10E), this corresponds for two samples from Puerto Rico, one from Laboratory colony and one captured in a bucket trap; 6.9 % of *H. zea* had ambiguous shape of distal margin (Figure 10F), this correspond for two samples from Puerto Rico captured in bucket traps. *Helicoverpa armigera* had 100 % of the samples as V-shape with the rounded apex (Figures 10A-C). There were founded overlaps between these species for the shape of distal margin too, but if one *Helicoverpa* has the distal apex in eighth sternite as V-shape with apex rounded probably is *H. armigera,* though is always necessary to check the number of lobes to confirm the identification. Complete data for qualitative variables is in Appendix 2.

Helicoverpa armigera and *H. zea* are species very related (Hardwick, 1965; Pogue, 2004). The most reliable morphological character to distinguish these species is the number of lobes; the other variables analyzed showed overlaps between these species, nevertheless they could be useful to support the identification.

3.4. Morphological comparison of *H. armigera, H. zea,* and their hybrids

In Table 2 and Figure 11 are showing the results of quantitative morphological variables analyzed. All *H. armigera* had one lobe in the vesica, *H. zea* and F1 hybrids (♂ *H. armigera* x ♀ *H. zea*) had three lobes (Figure 4). The statistical analysis of number of cornuti, depth of posterior excavation in eighth sternite (mm), length of aedeagus (mm), and length of valves (mm) situate *H. zea* with highest structures or more cornuti, then F1 hybrids (♂ *H. armigera* x ♀ *H. eighzea,* and *H. armigera* with smallest size of structures and minor number of cornuti (*P-value*<0.0001, Tukey test α=0.05). However, any of these characters is useful to distinguish hybrids due to the overlaps with *H. armigera* and *H. zea,* see boxplots of Figure 11, and minimum and maximum values for these variables in Table 2.

The results of the qualitative variables are resumed in Table 3. F1 hybrids (♂ *H. armigera* x ♀ *H. zea*) had rounded shape of distal apices in eighth sternite in 25.9 % of samples (Figure 8H), 37 % of F1 hybrids had distal apices with pointed shape (Figure 8G), and 37 % of hybrids had distal apices with ambiguous shape (Figure 8I), with this variable was difficult to infer if the F1 hybrids are closer to *H. zea* or *H. armigera* due to percentage

of ambiguity in hybrids. F1 hybrids showed 69.2 % of specimens with U-shape in proximal margin in the eighth sternite (Figure 9G); any F1 hybrid had the proximal margin as V-shape with apex flattened, and 30.8 % of F1 hybrids had an ambiguous shape of the proximal margin. It was difficult to infer with the shape of proximal margin if the F1 hybrids were closer to *H. zea* or *H. armigera* due to high variability in *H. armigera* in the shape of this structure. The shape of distal margin shows that F1 hybrids have 11.5 % of samples with U-shape (Figure 10G), 69.2 % of hybrids have the distal margin as V-shape with apex rounded (Figure 10H), and 19.2% of F1 hybrids have an ambiguous shape for this character (Figure 10I). It is possible to infer with the shape of distal margin that F1 hybrids are closer to *H. armigera* that have 100 % of the samples with V-shape with rounded apex in the distal margin.

In the principal components analysis (PCA) (Figure 12), the principal component 1 (PC1) explained 75.6 % of the variance, and principal component 2 (PC2) explained 10 % of the variance. The number of lobes, length of aedeagus, and number of cornuti had the higher loadings in the PC1, thus these variables had more influence in the model, number of lobes had the highest loading (0.46819), loadings are detailed in Appendix 3. The PCA showed that the F1 hybrid (♂ *H. armigera* x ♀ *H. zea*) is more related to *H. zea*, in this case the mother of the hybrids, only one hybrid was in the group of *H. armigera.* The number of lobes of hybrids equal to *H. zea* supported the result given by the PCA, and boxplots for variables number of cornuti, depth of posterior excavation in eighth sternite, length of aedeagus, and length of valves showed that measurements of hybrids were closer to *H. zea* than *H. armigera* (Figure 11). In other hybridization assays with Lepidoptera, analyzing the morphology of male genitalia, the F1 hybrids were also more similar to one of the parentals (Monti et al, 2001; Porter & Shapiro, 1990).

Using only morphology is very difficult to classify one *Helicoverpa* as a hybrid, because the F1 hybrids (♂ *H. armigera* x ♀ *H. zea*) obtained in this research had the same number of lobes as *H. zea*, and the other qualitative variables analyzed showed overlaps between *H. zea* and *H. armigera*. Laster & Sheng (1995) obtained F1 hybrids (♂ *H. armigera* x ♀ *H. zea*) but these authors did not describe the morphology of the hybrids.

Hardwick (1965) obtained F1 hybrids with the cross ♂ *H. zea* x ♀ *H. armigera*, the author reports that size of the wings of the hybrids was more related to *H. armigera*, the males had combined characters of *H. zea* and *H. armigera* , Hardwick (1965) reviewed other morphological characters in males not checked in this research; the author reported that females were more similar to *H. armigera*, in the current investigation females were not reviewed. Hardwick (1965) report that *Helicoverpa helenae* had similar morphology as F1 hybrids (♂ *H. zea* x ♀ *H. armigera)* and is a possibility that *H. helenae* could have a hybrid origin between these species.

4. Conclusions

In the cross ♂ *H. armigera* x ♀ *H. zea*, 21.4 % of females copulated, and 11.6 % of eggs were fertile, the pupal weight of hybrids was statistically equal as *H. zea, H. armigera* had the lowest pupal weight. In the cross ♂ *H. zea* x ♀ *H. armigera* obtained eggs (n= 21) were not fertile. *Helicoverpa armigera* had one lobe, while *H. zea* and F1 hybrids (♂ *H. armigera* x ♀ *H. zea*) presented three lobes, the number of lobes is the most reliable morphological character to identify and differentiate these species. In the remaining morphological variables analyzed were observed overlaps between *H. armigera* and *H. zea.* The statistical analysis of number of cornuti, length of valves, length and aedeagus, and depth of posterior excavation in the eighth sternite, showed that *H. zea* had the biggest structures or more cornuti, hybrids had smaller structures or less cornuti than *H. zea,* and *H. armigera* had the smallest structures and minor number of cornuti. The shape in structures in eight abdominal sternite had ambiguity in the species. It was not possible to infer if hybrids were closer to *H. zea* or *H. armigera* when considering the shape of distal apices or proximal margin due to ambiguity of the shapes. For the shape of distal margin, majority of *H. zea* showed U-shape, all *H. armigera* had V-shape with apex rounded, and 69.2 % of hybrids had distal margin as V-shape with apex rounded, in this case the hybrids were closer to *H. armigera* than *H. zea.* The principal component analysis was performed with quantitative variables, this analysis showed that F1 hybrids were morphologically more similar to *H. zea* than *H. armigera.* Finally, considering the characters analyzed in this study, it is very difficult to identify one *Helicoverpa* as hybrid using the morphology of male genitalia.

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Table 1: Aspects of the biology of *Helicoverpa armigera*, *Helicoverpa zea*, and hybrids.

Crosses	Fecundity		Pupal weight	
	% copulated ♀	% fertile eggs	n [1]	mg ± SD
♂ *H. armigera x* ♀ *H. armigera*	45.4	60.0	24	273.70±11.38 B[2]
♂ *H. zea* x ♀ *H. zea*	50.0	61.8	55	341.31±17.50 A
♂ *H. armigera x* ♀ *H. zea*	21.4	11.6	55	335.14±16.70 A
♂ *H. zea* x ♀ *H. armigera* [3]	14.2	0.0	-	-

1: n= number of insects analyzed
2: Means with different letter are significantly different at Tukey test α= 0.05, *P-value*= 0.0006
3: In this cross were not obtained fertile eggs

Table 2: Morphological comparison in the male genitalia of quantitative characters in *Helicoverpa armigera*, *Helicoverpa zea* and F1 hybrids *(♂ H. armigera x ♀ H. zea).*

Species	n[1]	Number of lobes		
H. zea	30	3		
F1 hybrid	23	3		
H. armigera	29	1		
		Number of cornuti		
	n	Mean ± SD		Min-max
H. zea	30	17.30 ± 1.44	A[2]	15 - 21
F1 hybrid	28	15.11 ± 0.99	B	13 - 17
H. armigera	30	12.07 ± 1.41	C	9 - 15
		P-value= <0.0001		
		Depth posterior excavation the eighth abdominal sternite		
	n	Mean ± SD (mm)		Min-max (mm)
H. zea	29	0.61 ± 0.08	A	0.42 - 0.76
F1 hybrid	26	0.52 ± 0.06	B	0.37 - 0.66
H. armigera	26	0.38 ± 0.05	C	0.29 – 0.52
		P-value= <0.0001		
		Length of aedeagus		
	n	Mean ± SD (mm)		Min-max (mm)
H. zea	29	5.19 ± 0.29	A	4.55 - 5.83
F1 hybrid	23	4.74 ± 0.36	B	3.89 - 5.61
H. armigera	27	4.19 ± 0.31	C	3.29 - 4.84
		P-value= <0.0001		
		Length of valves non- transformed data		
	n	Mean ± SD (mm)		Min-max (mm)
H. zea	30	4.75 ± 0.21		4.15 - 5.09
F1 hybrid	30	4.61 ± 0.21		4.03 – 4.99
H. armigera	29	4.16 ± 0.27		3.50 – 4.74
		Length of valves transformed data: x^3		
	n	Mean ± SD		Min-max
H. zea	30	107.92 ± 13.63	A	71.47 – 131.60
F1 hybrid	30	98.55 ± 12.54	B	65.26 – 123.90
H. armigera	29	72.69 ± 13.59	C	42.80- 106.40
		P-value= <0.0001		

1: n= number of specimens

2: Means with different letter are significantly different at Tukey test α= 0.05

Table 3: Morphological comparison in the male genitalia in the eighth abdominal sternite in *Helicoverpa armigera, H. zea,* and F1 hybrids *(♂ H. armigera x ♀ H. zea).* Showed data is the consensus of these variables qualified by five persons.

Species	Distal apices			
	n[1]	% Rounded	% Pointed	% Ambiguous[2]
H. zea	28	89.3	3.6	7.1
Hybrid	27	25.9	37.0	37.0
H. armigera	27	18.5	63.0	18.5
	Proximal margin			
	n	% U-shape	% V-shape apex flattened	% Ambiguous
H. zea	29	86.2	0.0	13.8
Hybrid	26	69.2	0.0	30.8
H. armigera	27	44.4	25.9	29.6
	Distal margin			
	n	% U-shape	% V-shape apex rounded	% Ambiguous
H. zea	29	86.2	6.9	6.9
Hybrid	26	11.5	69.2	19.2
H. armigera	26	0.0	100.0	0.0

1: n= number of insects analyzed

2: Considered ambiguos samples those qualified in different manner by two persons.

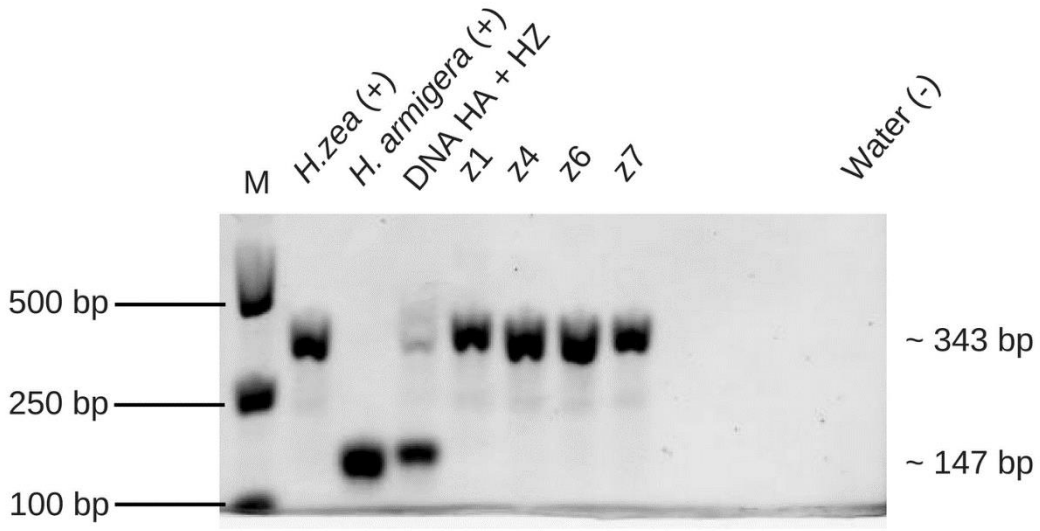

Figure 1: Agarose gel 1.2 % showing *Helicoverpa zea* amplicons from ITS1 region fragments of 343 bp from laboratory colony. *H. zea* (+) positive control; *H. armigera* (+) positive control; DNA HA + HZ mixture of *H. armigera*+ *H. zea;* z1, z4, z6, and z7 *H. zea* samples from the colony, Water (-) non-template control.

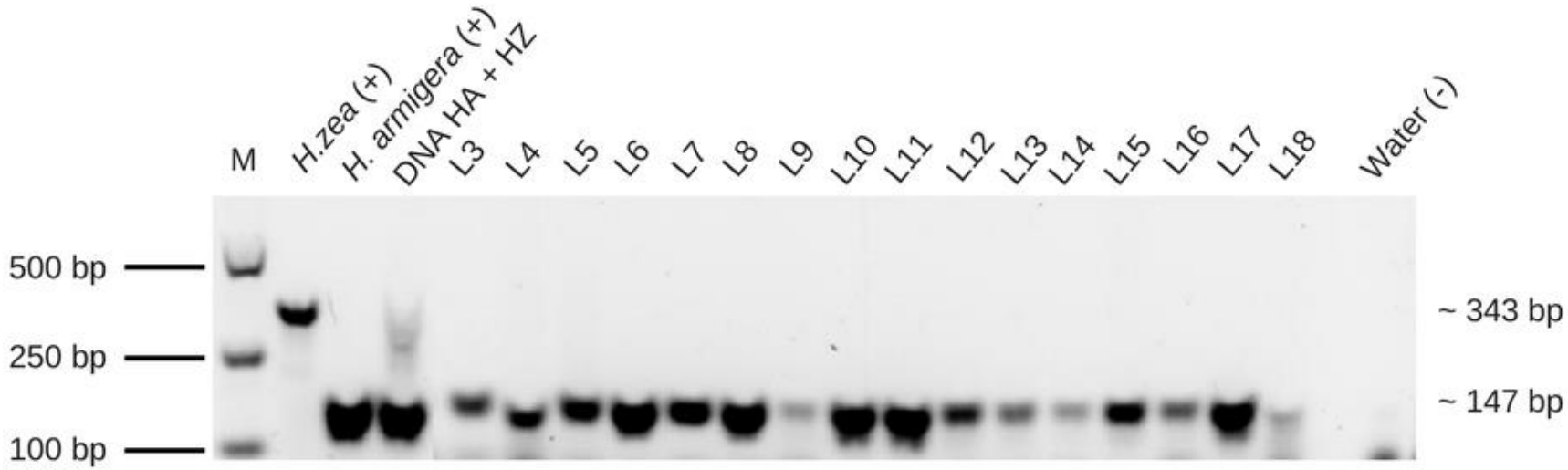

Figure 2: Agarose gel 1.2 % showing *Helicoverpa armigera* amplicons from ITS1 region fragments of 147 bp from laboratory colony. *H. zea* (+) positive control; *H. armigera* (+) positive control; DNA HA + HZ mixture of *H. armigera* + *H. zea;* L3-L18 *H. armigera* samples from the colony, Water (-) non-template control.

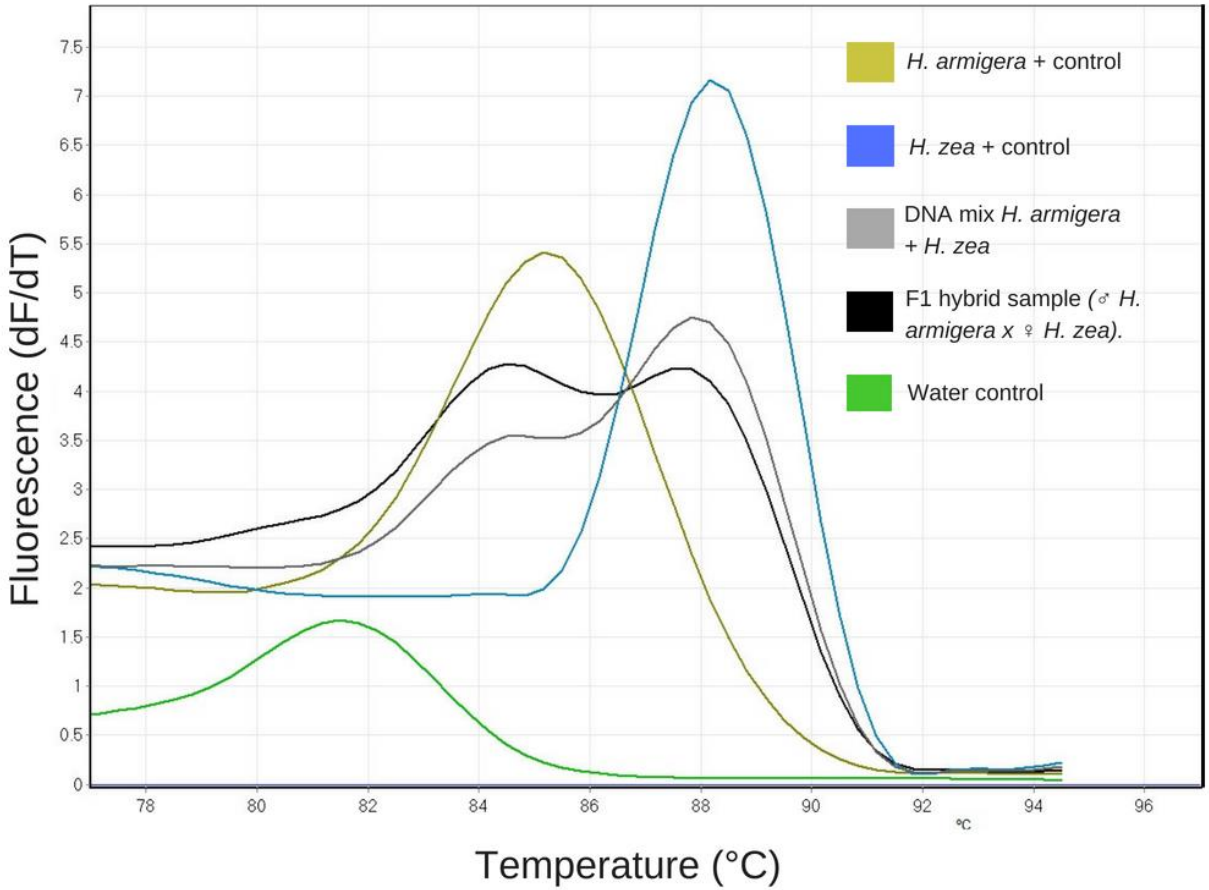

Figure 3: Melting curve analysis for Real Time PCR from primers ITS1 specific for *Helicoverpa armigera* and *Helicoverpa zea* (Perera et al., 2015), showing positive controls of *H. armigera*, *H. zea*, water control, DNA mixture of *H. armigera*+ *H. zea*, and F1 hybrid sample *(♂ H. armigera x ♀ H. zea*).

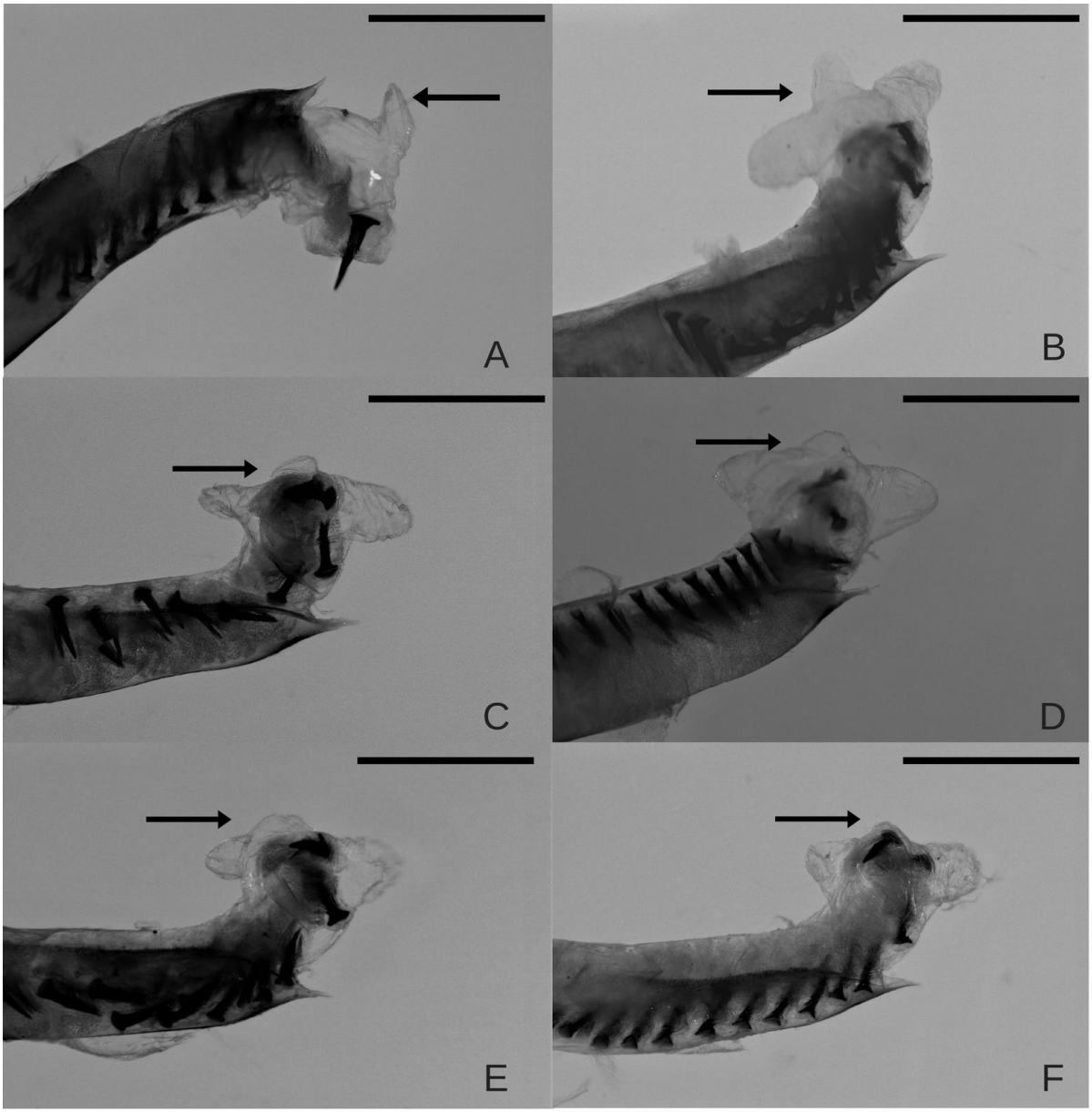

Figure 4: Images showing lobes in *Helicoverpa* male genitalia. **(A)** One lobe in *H. armigera* **(B)** Three lobes in *H. zea* **(C-F)** Three lobes in F1 hybrid *samples (♂ H. armigera x ♀ H. zea).* Length of bar= 1 mm.

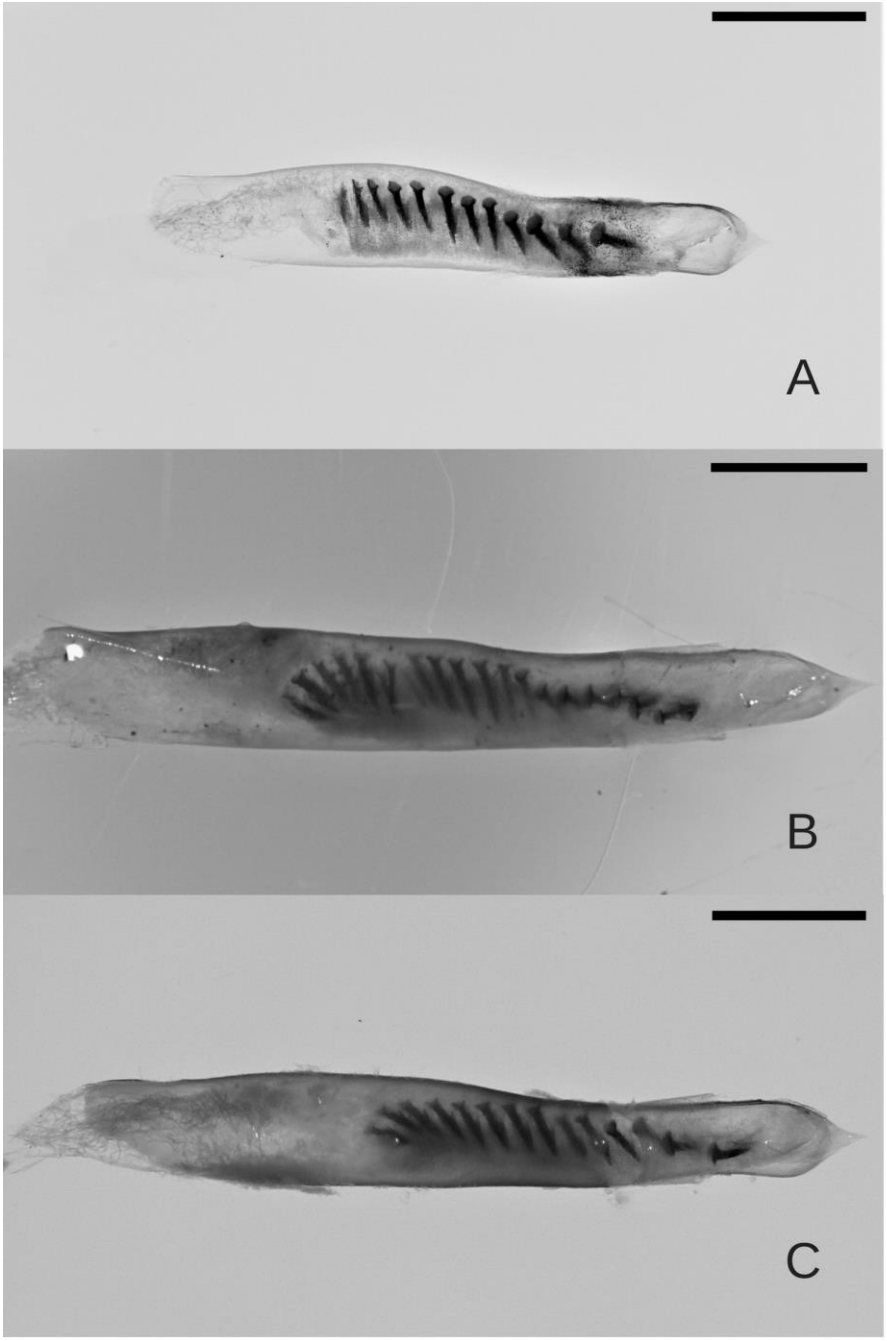

Figure 5: Images showing aedeagus in *Helicoverpa* male genitalia **(A)** *H. armigera* with 12 cornuti (black spines) in the aedeagus **(B)** *H. zea* with 19 cornuti in the aedeagus **(C)** F1 hybrid (♂ *H. armigera x* ♀ *H. zea)* with 14 cornuti in the aedeagus. Length of bar= 1 mm.

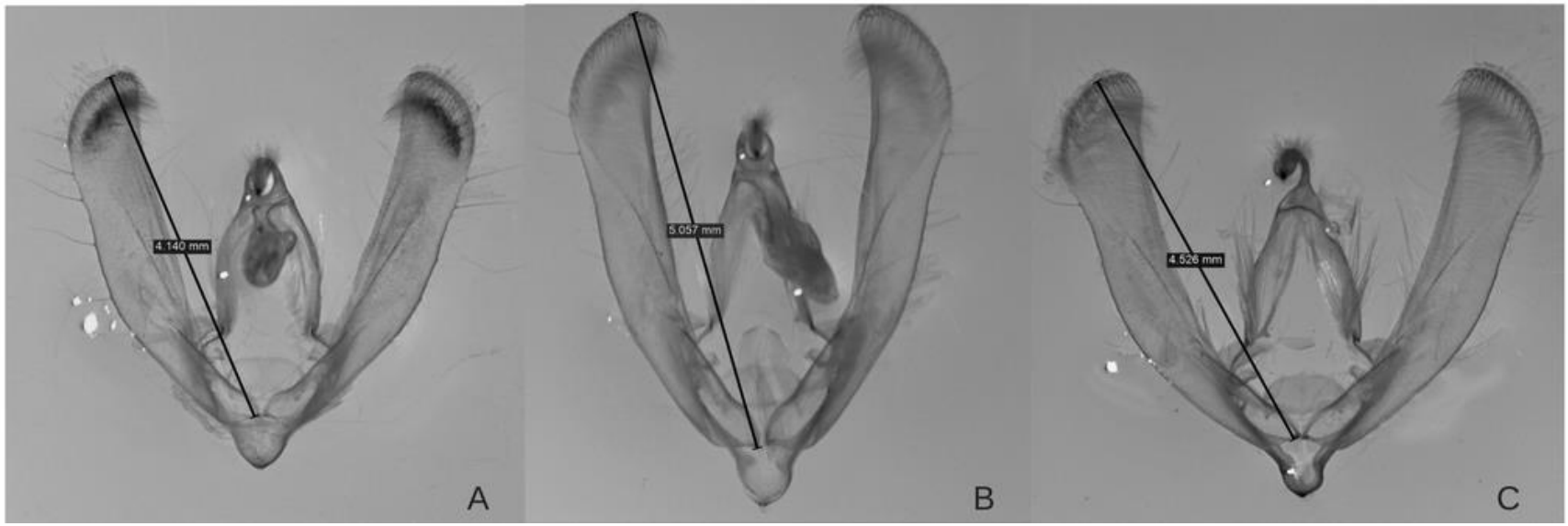

Figure 6: Images showing the length of valves (mm) in *Helicoverpa* male genitalia **(A)** Valves of *H. armigera* **(B)** Valves of *H. zea* **(C)** Valves of F1 hybrid (♂ *H. armigera x* ♀ *H. zea).*

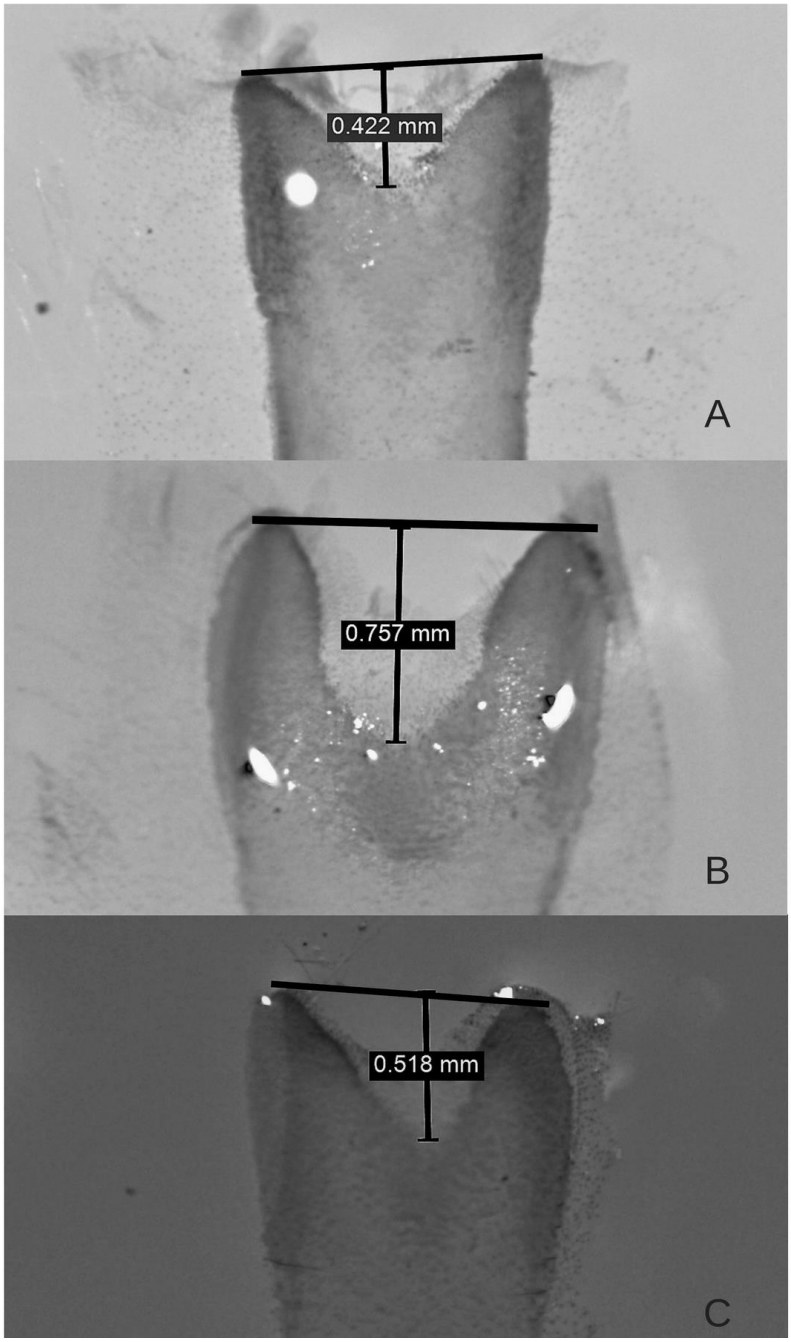

Figure 7: Images showing the depth of posterior excavation in the eighth abdominal sternite (mm) in *Helicoverpa* male genitalia **(A)** *H. armigera* **(B)** *H. zea* **(C)** F1 hybrid (♂ *H. armigera x* ♀ *H. zea).*

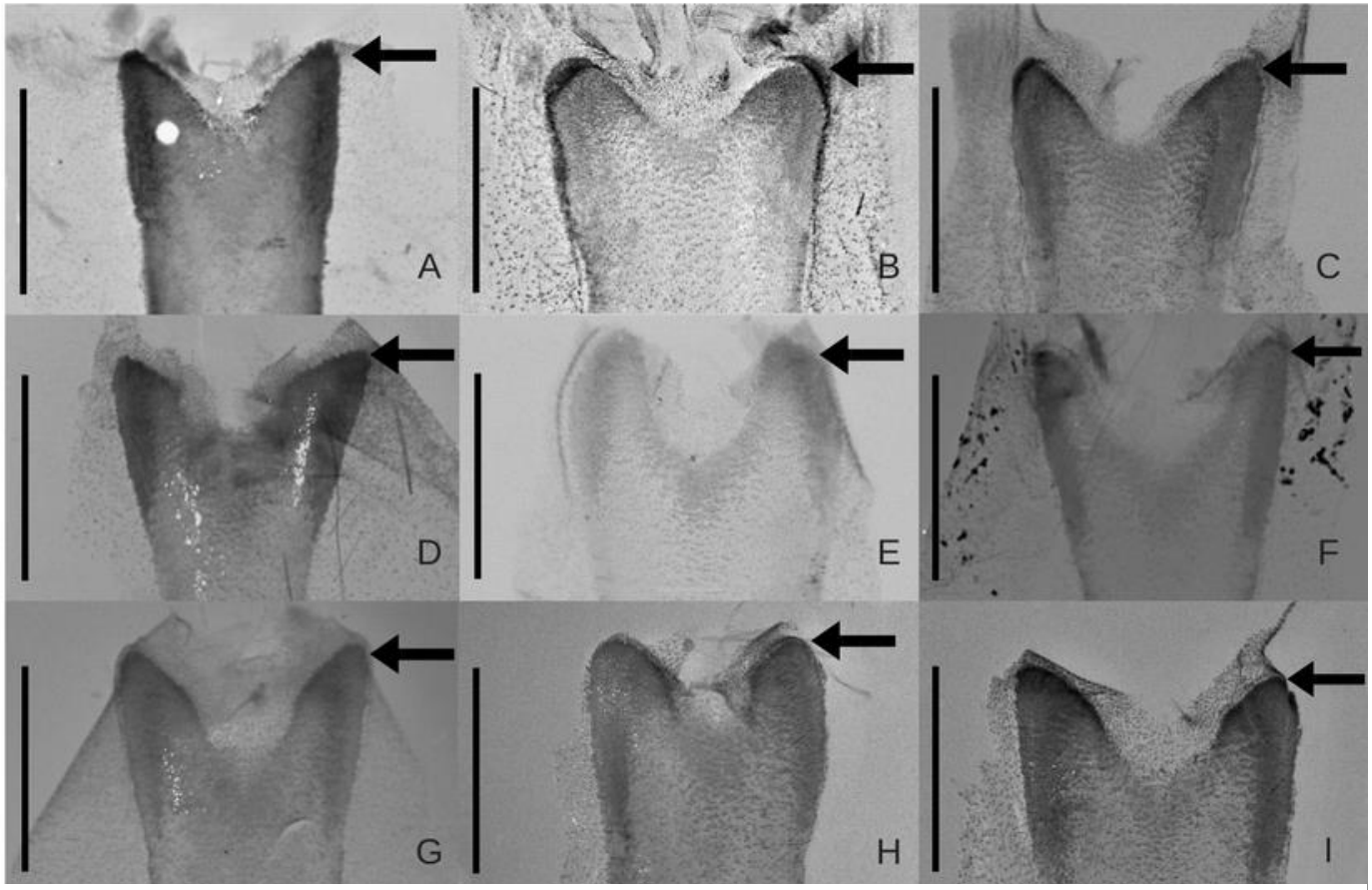

Figure 8: Images showing the shape of the distal apices in the eighth abdominal sternite in *Helicoverpa* male genitalia **(A)** *H. armigera* with pointed distal apices. **(B)** *H. armigera* with rounded distal apices **(C)** *H. armigera* with an ambiguous shape of distal apices. **(D)** *H. zea* with pointed distal apices **(E)** *H. zea* with rounded distal apices. **(F)** *H. zea* with an ambiguous shape of distal apices. **(G)** F1 hybrid *(♂ H. armigera x ♀ H. zea)* with pointed distal apices. **(H)** F1 hybrid *(♂ H. armigera x ♀ H. zea)* with rounded distal apices. **(I)** F1 hybrid *(♂ H. armigera x ♀ H. zea)* with an ambiguous shape of distal apices. Length bar = 1 mm.

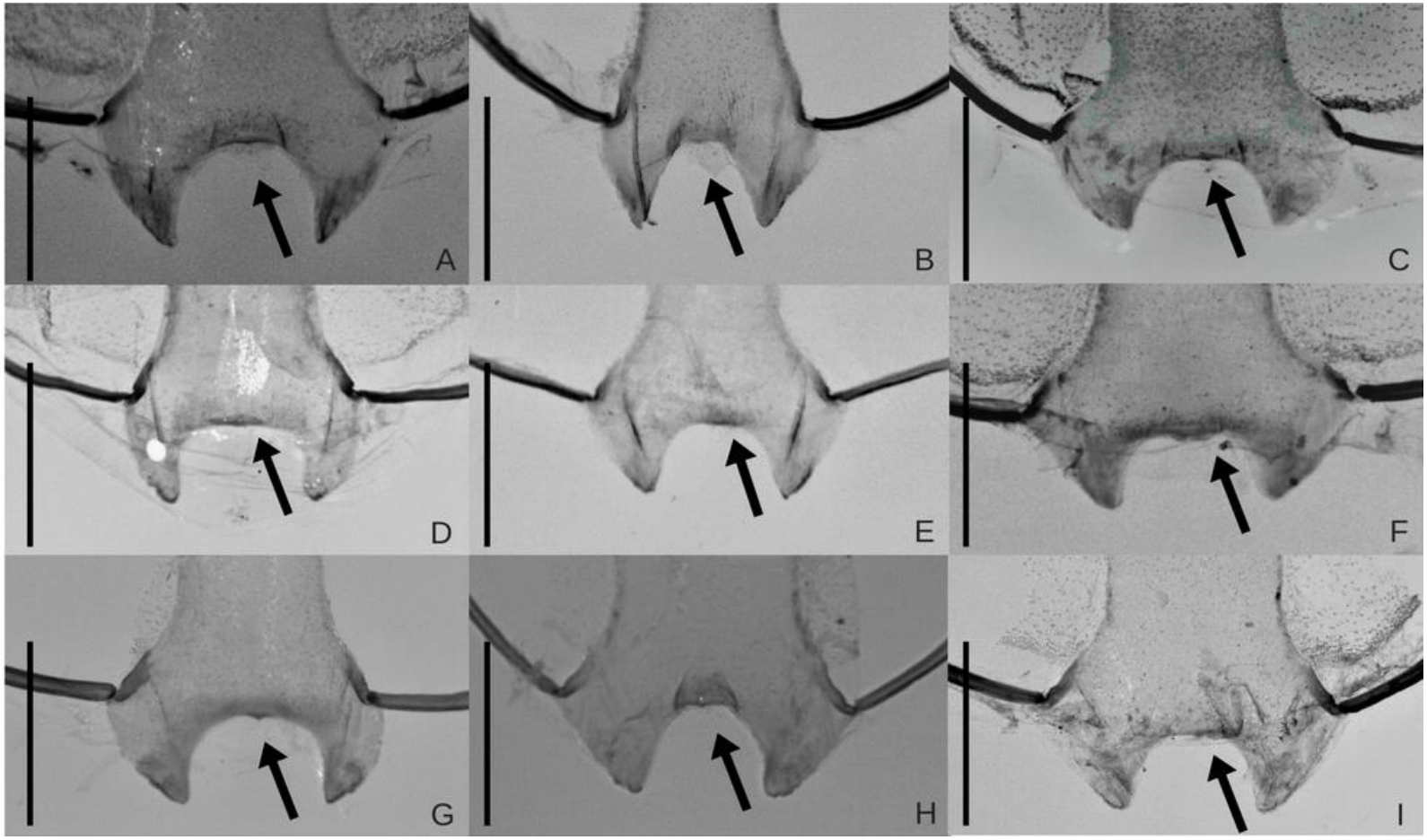

Figure 9: Images showing the shape of the proximal margin in the eight abdominal sternite in *Helicoverpa* male genitalia **(A)** *H. armigera* with U-shape proximal margin **(B)** *H. armigera* with V-shape and apex flattened in the proximal margin. **(C)** *H. armigera* with an ambiguous shape of the proximal margin. **(D)** *H. zea* with U-shape proximal margin. **(E-F)** *H. zea* with an ambiguous shape of the proximal margin. **(G)** F1 hybrid *(♂ H. armigera x ♀ H. zea)* with U-shape proximal margin **(H-I)** F1 hybrids *(♂ H. armigera x ♀ H. zea)* with an ambiguous shape of the proximal margin. Length bar= 1 mm.

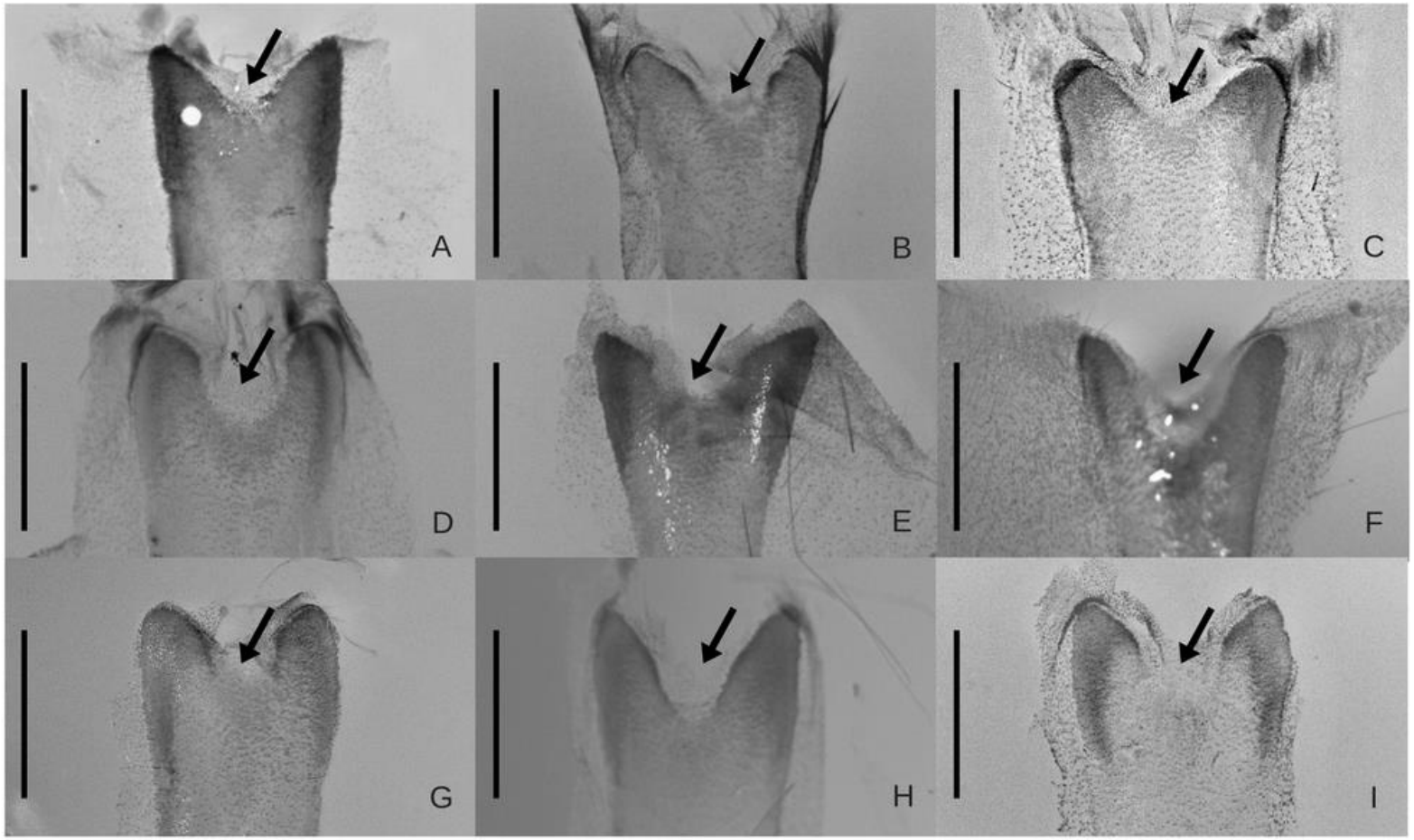

Figure 10: Images showing the shape of the distal margin in eighth abdominal sternite in *Helicoverpa* male genitalia. **(A-C)** *H. armigera* with V-shape with apex rounded in distal margin. **(D)** *H. zea* with U-shape distal margin **(E)** *H. zea* with V-shape with apex rounded in distal margin. **(F)** *H. zea* with an ambiguous shape in the distal margin. **(G)** F1 hybrid *(♂ H. armigera x ♀ H. zea)* with U-shape distal margin. **(H)** F1 hybrid *(♂ H. armigera x ♀ H. zea)* with V-shape with apex rounded in the distal margin. **(I)** F1 hybrid *(♂ H. armigera x ♀ H. zea)* with an ambiguous shape in distal margin. Length bar= 1 mm.

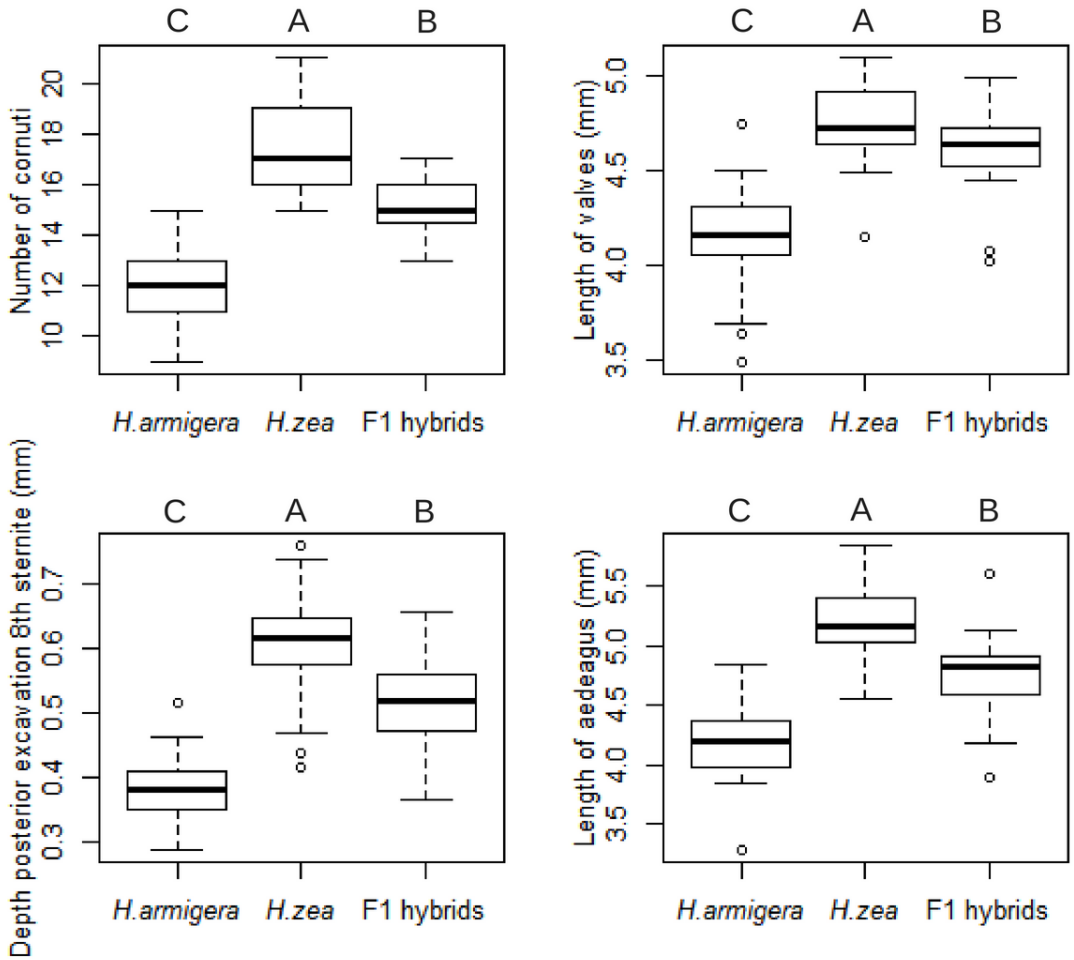

Figure 11: Boxplots for number of cornuti in aedeagus, length of valves (mm), depth of the posterior excavation in the eighth abdominal sternite (mm), and length of aedeagus (mm) in male genitalia of *H. armigera*, *H. zea*, and F1 hybrids *(♂ H. armigera x ♀ H. zea).* Different letters significantly different at Tukey test α= 0.05.

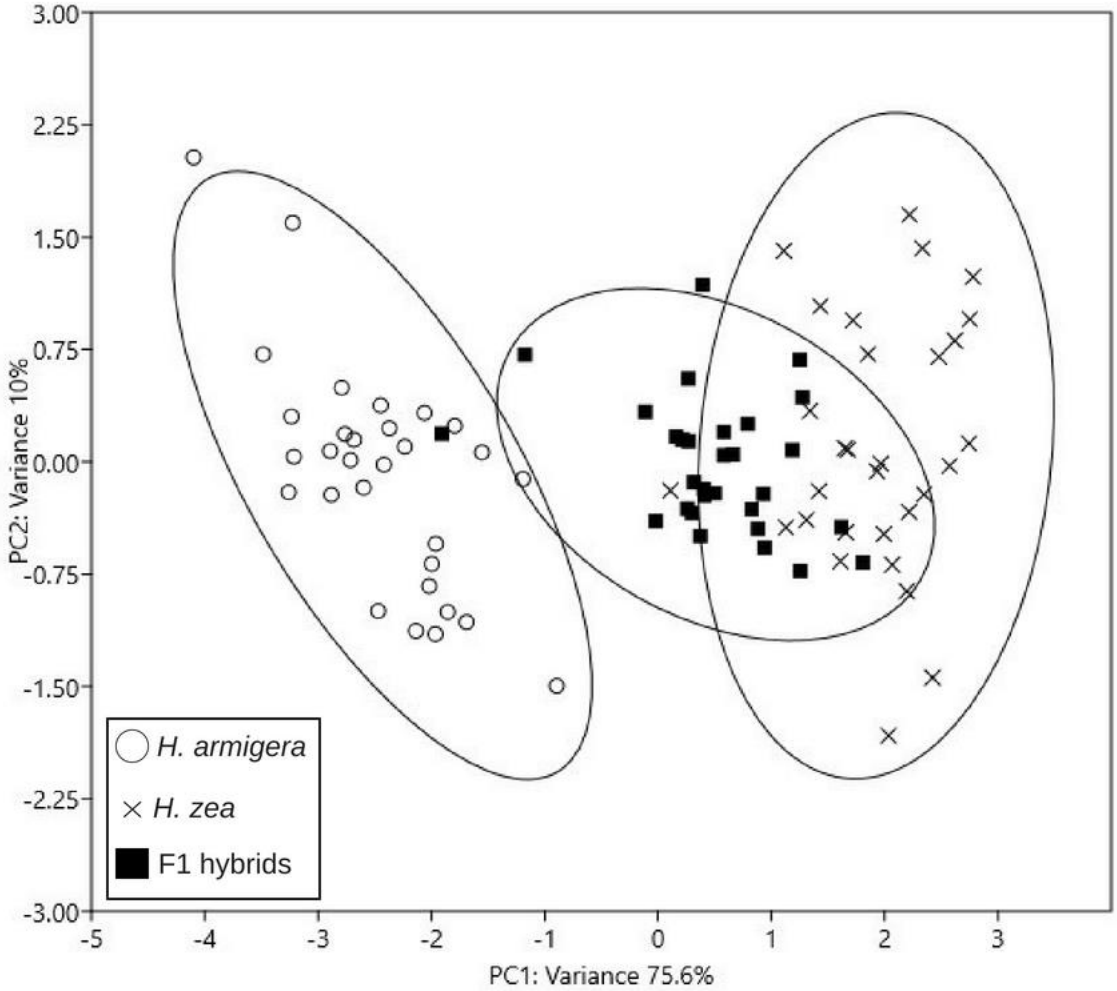

Figure 12: Principal component analysis of five morphological characters of *Helicoverpa* male genitalia: number of lobes, number of cornuti, depth of the posterior excavation in the eighth sternite (mm), length of valves (mm), and length of aedeagus (mm). *H. armigera* (n= 30), *H. zea* (n=30), and F1 hybrids *(♂ H. armigera x H. zea)* (n=30).

6. Appendices

Appendix 1: Quantitative characters for *Helicoverpa* males*:* F1 hybrids *(♂ H. armigera x ♀ H. zea)* (n=30), *H. armigera (*n= 30), and *H. zea* (n=30).

Origin	Specimen description	Number of lobes	Number of cornuti	Length of valves (mm)	Depth posterior excavation 8th sternite (mm)	Length of aedeagus (mm)
Laboratory	Hybrid	3	15	4.99		3.89
Laboratory	Hybrid		15	4.72	0.50	
Laboratory	Hybrid	3	15	4.55	0.41	4.60
Laboratory	Hybrid	3	15	4.55	0.53	4.75
Laboratory	Hybrid	3	16	4.78		
Laboratory	Hybrid	3	15	4.61	0.52	
Laboratory	Hybrid	3	15	4.72	0.50	5.05
Laboratory	Hybrid	3	16	4.71	0.55	5.61
Laboratory	Hybrid	3	15	4.52	0.44	4.95
Laboratory	Hybrid	3	15	4.72	0.66	
Laboratory	Hybrid		14	4.57	0.56	
Laboratory	Hybrid	3	15	4.85	0.52	5.13
Laboratory	Hybrid	3	16	4.51	0.50	4.86
Laboratory	Hybrid	3	16	4.93	0.56	5.03
Laboratory	Hybrid	3	15	4.53	0.47	4.86
Laboratory	Hybrid	3	14	4.84	0.62	4.82
Laboratory	Hybrid			4.71		
Laboratory	Hybrid	3	14	4.67	0.54	5.03
Laboratory	Hybrid			4.56	0.54	
Laboratory	Hybrid	3	17	4.69	0.45	4.86
Laboratory	Hybrid	3	14	4.56		4.86
Laboratory	Hybrid	3	16	4.71	0.61	4.77
Laboratory	Hybrid		17	4.50	0.46	4.81
Laboratory	Hybrid	3	14	4.48	0.56	4.57
Laboratory	Hybrid	3	15	4.65	0.56	4.70
Laboratory	Hybrid	3	15	4.69	0.49	4.65
Laboratory	Hybrid		14	4.07	0.47	4.38
Laboratory	Hybrid	3	15	4.45	0.47	4.39
Laboratory	Hybrid		13	4.03	0.37	4.28
Laboratory	Hybrid	3	17	4.46	0.55	4.19
Spain	*H. armigera*	1	14	4.74	0.38	4.84
Spain	*H. armigera*	1	13	4.46	0.32	4.50
Spain	*H. armigera*	1	12	4.40	0.38	4.68
Spain	*H. armigera*	1	12	4.50	0.39	4.66
Spain	*H. armigera*	1	15	4.30	0.29	4.41
Brazil	*H. armigera*	1	11		0.35	4.17

Continuation Appendix 1

Origin	Specimen description	Number of lobes	Number of cornuti	Length of valves (mm)	Depth posterior excavation 8th sternite (mm)	Length of aedeagus (mm)
Brazil	*H. armigera*	1	10	3.98	0.36	4.21
Brazil	*H. armigera*	1	12	3.79	0.35	4.15
Brazil	*H. armigera*	1	12	4.14	0.41	4.28
Brazil	*H. armigera*	1	10	4.30	0.40	3.98
Brazil	*H. armigera*	1	13	4.24	0.30	
Brazil	*H. armigera*	1	13	4.36		
Brazil	*H. armigera*	1	9	4.23	0.46	4.33
Brazil	*H. armigera*	1	12	4.05	0.40	4.18
Brazil	*H. armigera*	1	14	4.10	0.35	
Brazil	*H. armigera*	1	13	4.10	0.41	4.19
Brazil	*H. armigera*	1	11	3.64		3.91
Brazil	*H. armigera*	1	12	3.98	0.33	3.99
Brazil	*H. armigera*	1	12	4.14	0.37	3.92
Brazil	*H. armigera*	1	12	3.50	0.43	3.29
Brazil	*H. armigera*	1	14	4.16	0.39	4.16
Brazil	*H. armigera*	1	12	4.25	0.39	3.90
Brazil	*H. armigera*		11	4.07	0.42	4.21
Brazil	*H. armigera*	1	11	3.70	0.39	4.03
Brazil	*H. armigera*	1	13	4.09	0.37	3.97
Brazil	*H. armigera*	1	13	4.25	0.42	3.84
PR, bucket trap	*H. armigera*	1	13	4.38	0.37	4.39
PR, bucket trap	*H. armigera*	1	12	4.42	0.52	4.39
PR, bucket trap	*H. armigera*	1	9	4.01		4.25
PR, bucket trap	*H. armigera*	1	12	4.30		4.34
PR, Lab colony	*H. zea*	3	16	4.79	0.53	5.34
PR, Lab colony	*H. zea*	3	19	5.00	0.62	5.14
PR, Lab colony	*H. zea*	3	18	4.15	0.62	4.97
PR, Lab colony	*H. zea*	3	16	4.88	0.63	5.16
PR, Lab colony	*H. zea*	3	17	5.05	0.44	5.65
PR, Lab colony	*H. zea*	3	17	4.66	0.65	4.62
PR, Lab colony	*H. zea*	3	18	5.06		
PR, Lab colony	*H. zea*	3	16	4.97	0.58	5.54
PR, Lab colony	*H. zea*	3	19	4.86	0.68	4.98
PR, Lab colony	*H. zea*	3	16	4.92	0.63	5.41
PR, Lab colony	*H. zea*	3	21	4.66	0.64	5.18
PR, Lab colony	*H. zea*	3	19	4.59	0.73	5.00
PR, Lab colony	*H. zea*	3	19	4.56	0.74	4.87
PR, Lab colony	*H. zea*	3	17	4.99	0.63	5.25
PR, Lab colony	*H. zea*	3	19	4.76	0.73	5.22

Continuation Appendix 1

Origin	Specimen description	Number of lobes	Number of cornuti	Length of valves (mm)	Depth posterior excavation 8th sternite (mm)	Length of aedeagus (mm)
PR, Lab colony	*H. zea*	3	18	4.64	0.47	5.11
PR, Lab colony	*H. zea*	3	17	4.52	0.57	5.07
PR, Lab colony	*H. zea*	3	17	4.71	0.53	5.07
PR, Lab colony	*H. zea*	3	19	4.85	0.65	5.43
PR, Lab colony	*H. zea*	3	17	4.72	0.67	4.95
PR, Lab colony	*H. zea*	3	19	4.70	0.76	5.20
PR, bucket trap	*H. zea*	3	16	4.54	0.42	4.55
PR, bucket trap	*H. zea*	3	16	4.72	0.61	5.16
PR, bucket trap	*H. zea*	3	18	4.49	0.64	5.04
PR, bucket trap	*H. zea*	3	16	4.64	0.61	5.31
PR, bucket trap	*H. zea*	3	15	5.09	0.57	5.83
PR, bucket trap	*H. zea*	3	16	4.61	0.63	5.59
PR, bucket trap	*H. zea*	3	16	4.73	0.50	5.03
PR, bucket trap	*H. zea*	3	16	4.83	0.59	5.46
PR, bucket trap	*H. zea*	3	16	4.91	0.58	5.47

Appendix 2: Qualitative characters for *Helicoverpa* males*:* F1 hybrids *(♂ H. armigera x ♀ H. zea)* (n=30), *H. armigera* (n= 30), and *H. zea* (n=30), qualified by five persons.

Origin	Specimen description	Distal apex: rounded or pointed (R or P)						Shape proximal margin: U or V with apex flattened (U or V)						Shape distal margin: U or V with apex rounded (U or V)					
		Person 1	Person 2	Person 3	Person 4	Person 5	Consensus	Person 1	Person 2	Person 3	Person 4	Person 5	Consensus	Person 1	Person 2	Person 3	Person 4	Person 5	Consensus
Laboratory	Hybrid	R	R	P	P	P	A[1]	U	U	V	U	V	A	V	U	V	V	V	V*
Laboratory	Hybrid	P	R	P	P	P	P*	V	U	V	U	V	A	V	U	V	V	V	V*
Laboratory	Hybrid	P	R	R	P	P	A	V	U	U	U	V	A	V	U	V	U	V	A
Laboratory	Hybrid	P	R	R	P	P	A	V	U	V	U	V	A	V	U	V	V	V	V*
Laboratory	Hybrid	P	R	P	P	P	P*	U	U	V	U	V	A	V	U	V	V	V	V*
Laboratory	Hybrid	R	R	R	R	R	R	U	U	U	U	U	U	U	U	U	U	U	U
Laboratory	Hybrid	R	R	P	R	R	R*	U	U	V	U	U	U*	U	U	V	U	V	A
Laboratory	Hybrid	R	R	P	R	P	A	U	U	U	U	V	U*	V	U	V	V	V	V*
Laboratory	Hybrid	R	R	P	R	R	R*	U	U	V	U	U	U*	V	U	V	V	V	V*
Laboratory	Hybrid	P	R	P	P	P	P*	U	U	U	U	U	U	V	U	V	V	V	V*
Laboratory	Hybrid	R	R	R	R	P	R*	U	U	U	U	U	U	U	U	U	U	V	U*
Laboratory	Hybrid	P	R	P	R	P	A	U	U	V	U	U	U*	V	U	V	V	V	V*
Laboratory	Hybrid	R	R	R	R	R	R	U	U	U	U	U	U	U	U	V	U	V	A
Laboratory	Hybrid	P	R	P	P	R	A	NA[2]	NA	NA	NA	NA		V	U	V	U	V	A
Laboratory	Hybrid	P	R	P	R	P	A	U	U	V	U	U	U*	V	U	V	V	V	V*
Laboratory	Hybrid	R	R	R	R	R	R	U	U	U	U	U	U	U	U	U	U	V	U*
Laboratory	Hybrid	R	R	R	R	P	R*	U	U	U	U	U	U	V	U	V	V	V	V*
Laboratory	Hybrid	P	R	P	P	R	A	U	U	U	U	U	U	NA	NA	NA	NA	NA	
Laboratory	Hybrid	P	P	P	P	P	P	U	U	U	U	V	U*	V	U	V	V	V	V*
Laboratory	Hybrid	R	R	R	R	P	P *	U	U	U	U	U	U	U	U	U	V	V	A

Continuation Appendix 2

Origin	Specimen description	Distal apex: rounded or pointed (R or P)						Shape proximal margin: U or V with apex flattened (U or V)						Shape distal margin: U or V with apex rounded (U or V)					
		Person 1	Person 2	Person 3	Person 4	Person 5	Consensus	Person 1	Person 2	Person 3	Person 4	Person 5	Consensus	Person 1	Person 2	Person 3	Person 4	Person 5	Consensus
Laboratory	Hybrid	P	P	P	P	P	P	U	U	U	U	U	U	V	U	V	V	V	V*
Laboratory	Hybrid	P	P	P	P	P	P	V	V	V	U	U	A	V	U	V	V	V	V*
Laboratory	Hybrid	P	P	P	P	P	P	U	U	U	U	U	U	V	V	V	V	V	V
Laboratory	Hybrid	R	P	P	R	P	A	V	V	V	U	U	A	V	V	V	V	V	V
Laboratory	Hybrid	P	P	P	P	P	P	U	U	U	U	U	U	V	U	V	V	V	V*
Laboratory	Hybrid	R	R	P	R	P	A	U	U	U	U	U	U	V	V	V	V	V	V
Laboratory	Hybrid	P	P	P	P	P	P	U	U	V	V	V	A	V	V	V	V	V	V
Spain	*H. armigera*	P	P	P	P	P	P	V	V	V	V	V	V	V	V	V	V	V	V
Spain	*H. armigera*	P	P	P	P	P	P	U	U	U	U	U	U	V	V	V	V	V	V
Spain	*H. armigera*	R	P	P	P	P	P	U	U	V	U	U	U*	V	V	V	V	V	V
Spain	*H. armigera*	P	P	P	P	P	P	U	U	U	U	U	U	V	V	V	V	V	V
Spain	*H. armigera*	P	P	P	P	P	P	V	V	V	U	U	A	V	V	V	V	V	V
Brazil	*H. armigera*	R	R	R	P	P	A	U	U	U	V	V	A	V	V	V	V	V	V
Brazil	*H. armigera*	P	P	R	P	P	P *	V	U	U	V	V	A	V	V	V	V	V	V
Brazil	*H. armigera*	R	R	R	R	P	R*	U	U	V	U	V	A	V	V	V	V	V	V
Brazil	*H. armigera*	R	R	R	R	P	R*	U	U	U	U	V	U*	NA	NA	NA	NA	NA	
Brazil	*H. armigera*	P	P	P	P	P	P	V	V	V	U	V	V*	V	V	V	V	V	V
Brazil	*H. armigera*	P	P	P	P	P	P	V	U	V	U	V	A	V	V	V	V	V	V
Brazil	*H. armigera*	P	P	P	P	P	P	V	V	V	U	V	V*	V	V	V	V	V	V
Brazil	*H. armigera*	P	P	R	R	P	A	V	V	V	U	V	V*	V	V	V	V	V	V
Brazil	*H. armigera*	P	P	R	P	P	P *	U	U	U	U	V	U*	V	V	V	V	V	V
Brazil	*H. armigera*	R	R	R	R	P	R*	V	V	V	V	V	V	V	V	V	V	V	V

Continuation Appendix 2

Origin	Specimen description	Distal apex: rounded or pointed (R or P)						Shape proximal margin: U or V with apex flattened (U or V)						Shape distal margin: U or V with apex rounded (U or V)					
		Person 1	Person 2	Person 3	Person 4	Person 5	Consensus	Person 1	Person 2	Person 3	Person 4	Person 5	Consensus	Person 1	Person 2	Person 3	Person 4	Person 5	Consensus
Brazil	*H. armigera*	R	R	R	R	P	R*	U	U	U	U	V	U*	V	V	V	V	V	V
PR, bucket trap	*H. armigera*	P	P	R	P	P	P *	U	U	U	U	U	U	V	V	V	V	V	V
PR, bucket trap	*H. armigera*	P	P	P	P	P	P	V	V	V	V	V	V	V	V	V	V	V	V
Brazil	*H. armigera*	R	R	P	R	P	A	V	U	V	U	V	A	V	V	V	V	V	V
Brazil	*H. armigera*	R	P	P	R	P	A	U	U	V	U	U	U*	V	V	V	V	V	V
Brazil	*H. armigera*	P	P	P	P	P	P	V	V	V	U	V	V*	V	V	V	V	V	V
Brazil	*H. armigera*	P	P	P	P	P	P	U	U	U	U	V	U*	V	V	V	V	V	V
Brazil	*H. armigera*	R	R	R	R	P	R*	U	U	U	U	V	U*	V	V	V	V	V	V
Brazil	*H. armigera*	P	P	P	R	P	P *	U	U	U	U	U	U	V	V	V	V	V	V
Brazil	*H. armigera*	R	R	P	R	P	A	U	U	V	U	U	U*	V	V	V	V	V	V
Brazil	*H. armigera*	P	P	P	P	P	P	U	V	V	U	U	A	V	V	V	V	V	V
Brazil	*H. armigera*	P	P	P	P	P	P	U	V	V	U	V	A	V	V	V	V	V	V
PR, Lab colony	*H. zea*	R	R	R	R	R	R	U	U	U	V	V	A	U	U	U	U	U	U
PR, Lab colony	*H. zea*	R	R	R	R	R	R	U	U	U	U	U	U	U	U	U	U	U	U
PR, Lab colony	*H. zea*	R	R	R	R	R	R	U	U	V	U	V	A	U	U	U	U	U	U
PR, Lab colony	*H. zea*	R	R	R	R	R	R	A	U	A	U	V	U*	U	U	U	U	U	U
PR, Lab colony	*H. zea*	NA	NA	NA	NA	NA		U	U	U	U	V	U*	U	U	U	U	U	U
PR, Lab colony	*H. zea*	R	R	R	R	R	R	U	U	U	U	V	U*	U	U	U	U	U	U
PR, Lab colony	*H. zea*	R	R	R	R	R	R	U	U	U	U	V	U*	U	U	U	U	U	U
PR, Lab colony	*H. zea*	R	R	R	R	R	R	U	U	U	U	V	U*	U	U	U	U	U	U
PR, Lab colony	*H. zea*	R	R	R	R	R	R	U	U	U	U	V	U*	U	U	U	U	U	U
PR, Lab colony	*H. zea*	R	R	R	R	R	R	U	U	U	U	V	U*	U	U	U	U	U	U

Continuation Appendix 2

Origin	Specimen description	Distal apex: rounded or pointed (R or P)						Shape proximal margin: U or V with apex flattened (U or V)						Shape distal margin: U or V with apex rounded (U or V)					
		Person 1	Person 2	Person 3	Person 4	Person 5	Consensus	Person 1	Person 2	Person 3	Person 4	Person 5	Consensus	Person 1	Person 2	Person 3	Person 4	Person 5	Consensus
PR, Lab colony	*H. zea*	R	R	R	R	R	R	U	U	U	U	V	U*	U	U	U	U	U	U
PR, Lab colony	*H. zea*	R	R	R	R	R	R	V	U	U	V	V	A	U	U	U	U	U	U
PR, Lab colony	*H. zea*	R	R	R	R	R	R	U	U	U	U	V	U*	U	U	U	U	U	U
PR, Lab colony	*H. zea*	R	R	R	P	R	R*	U	U	U	U	V	U*	U	U	U	U	U	U
PR, Lab colony	*H. zea*	R	R	R	R	R	R	U	U	U	U	V	U*	U	U	U	U	U	U
PR, Lab colony	*H. zea*	R	R	R	R	R	R	U	U	U	U	V	U*	U	U	U	U	U	U
PR, Lab colony	*H. zea*	R	R	R	R	R	R	U	U	U	U	V	U*	U	U	U	U	U	U
PR, Lab colony	*H. zea*	R	R	R	R	R	R	U	U	U	U	V	U*	V	V	V	V	U	V*
PR, Lab colony	*H. zea*	R	R	R	R	R	R	U	U	U	U	V	U*	U	U	U	U	U	U
PR, Lab colony	*H. zea*	R	R	R	R	R	R	U	U	U	U	V	U*	U	U	U	U	U	U
PR, bucket trap	*H. zea*	R	R	R	R	R	R	U	U	U	U	U	U	V	U	V	U	V	A
PR, bucket trap	*H. zea*	R	R	R	R	R	R	U	U	V	U	U	U*	U	U	U	U	V	U*
PR, bucket trap	*H. zea*	P	P	P	P	P	P	U	V	U	U	U	U*	V	V	V	V	V	V
PR, bucket trap	*H. zea*	R	R	R	R	R	R	U	U	U	U	U	U	U	U	U	U	U	U
PR, bucket trap	*H. zea*	R	R	R	R	R	R	U	U	U	U	U	U	U	U	U	U	U	U
PR, bucket trap	*H. zea*	P	R	P	P	R	A	V	U	V	U	U	A	U	U	V	U	U	U*
PR, bucket trap	*H. zea*	R	R	R	R	R	R	U	U	U	U	U	U	U	U	U	U	U	U
PR, bucket trap	*H. zea*	P	R	P	P	R	A	U	U	U	U	U	U	U	U	V	U	V	A
PR, bucket trap	*H. zea*	R	R	R	R	R	R	U	U	U	U	U	U	U	U	U	U	U	U

* One person qualified in different manner.

1: A= ambiguous result, two persons qualified in different manner.

Appendix 3: Loadings for Principal Component Analysis for study of morphological comparison of *Helicoverpa armigera, H. zea,* and F1 hybrids *(♂ H. armigera x ♀ H. zea).*

Character	PC 1	PC 2	PC 3	PC 4	PC 5
Number of lobes	0.46819	0.1352	-0.32148	-0.41701	-0.69661
Number of cornuti	0.45441	0.23714	-0.6302	0.44688	0.37474
Length of valves (mm)	0.44381	-0.54024	0.037454	-0.52109	0.48809
Depth posterior excavation 8 sternite (mm)	0.42308	0.64843	0.59687	-0.091502	0.18952
Length of aedeagus (mm)	0.44536	-0.4617	0.37663	0.58863	-0.31646