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Morphological, molecular and biological characterization of *Spodoptera frugiperda* (J.E. Smith) from Rice in Northern Luzon, Philippines

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Abstract The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) is a new invasive pest of corn in the Philippines.—The larvae were identified which based on morphological characteristics and through molecular techniques. *Cytochrome C oxidase I* A region (*COI*A) amplification and comparison with nucleotide sequences in the GenBank was used to identify. All larvae collected from Barangay Annanuman, San Pablo, Isabela showed morphological characteristics identified as *S. frugiperda*. Analysis of the *COI*A gene region revealed the presence of both the corn strain (CS) and rice strain (RS) in the samples. Biological studies on young leaves of corn (IPB var 6) showed that the eggs were dome-shaped, laid in clusters, covered with cottony scales, and were light-colored, but turned dark or black when about to hatch. The neonate larva was yellowish and turned brown or dark green to purplish green at maturity. The larvae passed through six instars. Total larval period of all instars was completed in 10.18±0.99 and 9.62±0.98 days for the male and the female, respectively. The pupal period was 8.43±0.51 days for the male and 7.23±0.6 days for the female. Total development period was 20.61±1.0 days for the male and 18.85±1.26 days for the female. Fecundity of females that emerged from heavy pupae of 1,857.75±858.07 eggs per female did not differ from females that emerged from lighter pupae ($\bar{X}$= 1,390±303.03 eggs). Hatchability of eggs was 74.15±23.16 percent (from heavy pupae) and 66.02±21.82 percent (from light pupae). Male adults that emerged from light pupae lived significantly longer than those from heavy pupae. The present study confirmed the presence of both RS and CS in rice from Isabela. This is the first report of occurrence of the RS and CS of *S. frugiperda* in rice in the Philippines.

Keywords: *Spodoptera frugiperda*, Molecular identification, *COI*A, Rice (RS) and Corn (CS) strains

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Introduction

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), is an invasive and highly polyphagous species native to the Americas. It is the most economically important lepidopterous pest of maize in South and Central America (Sparks, 1979) which migrates northward annually in the fall season to infest maize crops (Prasanna *et al.,* 2022).

In January 2016, *S. frugiperda* was first reported outside its native range in West Africa (Goergen *et al.,* 2016). By January 2018, the pest has invaded majority of the African countries (Prasanna *et al.,* 2018; Montezano *et al.,* 2018; Igyuve *et al.,* 2018). Its impact can be seen from the fact that corn is the main staple crop grown by small farmers in sub-Saharan Africa (Macauley, 2015), of which 90% of production is used for food (Igyuve, 2018; Pashley, 1988) .

In Asia, FAW was first recorded in the southern Indian state of Karnataka in May 2018 (Sharanabasappa *et al.,* 2018; Shylesha *et al.,* 2018) and rapidly spread across the Asia-Pacific region, affecting Yemen, Bangladesh, Myanmar, China, Thailand, Sri Lanka, Nepal, Philippines, Vietnam, Indonesia, Australia, South Korea, Cambodia, Papua New Guinea, Timor Leste, New Caledonia, Jordan, Syria, and the United Arab Emirates (reviewed by Prasanna *et al.,* 2022).

FAW was first reported in the Philippines in 2019 (IPPC, 2019; Navasero *et al.,* 2019), infesting mostly conventional maize in 8,000 ha as of June 2020 and reaching 12,000 ha by October 2020 (DA Communications Group, 2020). The Philippine government projected a P20-billion economic loss and predicted 1.6 million metric tons of harvest from 2.5 million ha of corn areas to be affected by FAW (Miraflor, 2020). The severity of the FAW infestation in the country also affected feed millers, food processors, livestock and poultry raisers, traders and consolidators, and consumers.

Spodoptera frugiperda affects maize, sorghum, sugar cane, forage grasses, peat grasses, rice, cotton and peanuts. It is a voracious pest in its invading countries (Knipling, 1980; Pashley, 1986; Levy *et al*. (2002). Montezano *et al*. (2018) reported 353 plant species as hosts in America, distributed in 76 families, mainly Poaceae, Asteraceae and Fabaceae.Reports showed that larvae of *S. fugiperda* feed on both leaves and fruit structures of host plants.Its development is strongly influenced by host plant and temperature.In young maize, larvae feed on the surface of leaves only white one's papery patches called window panes. On the other hand, older larvae with stronger mandibles consume more tissue and can cut large sections of plant tissue high in silica, including seedlings, leaves, tassels, cobs, shells, and

developing nuclei (Pogue, 2002; Goergen *et al.*, 2016; Navasero and Navasero, 2020).

S. frugiperda is comprised of two genetically and behaviorally separated strains that occur sympatrically throughout North and South America (Pashley, 1986; Prasanna *et al.*, 2022). The corn strain (CS) was reported to damage maize, sorghum, and cotton, whereas the rice strain (RS) predominantly infests rice, alfalfa, pasture, and forage grasses. These strains of FAW are morphologically indistinguishable, but through mitochondrial cytochrome *cytochrome C oxidase subunit I* (*COI*) gene, they can be consistently differentiated and identified based on their haplotypes. In America, however, cryptic host strains have previously been distinguished by differences in protein composition, nuclear DNA restriction fragment length polymorphism (RELP) (Pashley *et al*., 1985; Lu *et al*., 1992; Lu and Adang, 1996), and amplified fragment-length polymorphism (AFLP) to detect differences among FAW strains, including hybrids (McMichael and Prowell, 1999). The strain distribution of armyworm populations that invaded sub-Saharan Africa was analyzed by Nagoshi *et al.* (2019). *S. frugiperda*, an invasive insect pest, has a remarkable dispersal ability that is part of its life history strategy (Johnson, 1987). In its annual migration, it can extend more than 2,000 km from its endemic range in the warmer parts of the New World across the entire United States to Canada in the north and reach northern parts of Argentina and Chile in the south (Pair *et al*., 1986).

The biology of *S. frugiperda* has been reviewed by Sparks (1979), including its distribution, economic importance, seasonal abundance, host plants, life history, and its natural, cultural, genetic, and chemical control (Andrews, 1998). They reported that the biology of FAW varies depending on larval development in specific hosts and the temperature of the land. In the Philippines, Navasero and Navasero (2020) reported that the egg hatched in 2 to 3 days, with a larval period of 14.73 days for males and 14.4 days for females. They also reported a significantly shorter pupal period of 8.5 days for the female and 9.0 days for the male. Therefore, the females emerged before the males. Fertile females laid eggs ranging from 800 to 1,639; fertile eggs accounted for 73.9% of the eggs laid. Males lived slightly longer than females.

The investigation is reported to be the first time that the population of *S. frugiperda* occurring on rice using both morphological and molecular analyses. It also examined growth and development of larvae when shifted from rice to corn as host plant, as well as the effects of pupal weight on fecundity, longevity of adults, and percent egg hatch.

Materials and methods

Test insects

The occurrence of FAW was observed in rice seedbed in three provinces in Luzon Island, Philippines during detection surveys from May to June 2021. FAW was noted in isolated packets in Barangay Pateng, Gonzaga, Cagayan (May 17, 2021), Sta. Ana, Cagayan (May 20, 2021), Enrile, Cagayan (May 21, 2021), Cobarroquis, Quirino (May 26, 2021), San Pablo, Isabela (May 27, 2021), Maddela, Quirino (May 28, 2021), and Barangay Annanuman, San Pablo, Isabela (June 2, 2021) (Figure 1). The field symptoms were observed and documented (Figure 2). Larvae collected from Barangay Annanuman were brought to the laboratory for observation and rearing. Upon emergence, adult males and females were released in Mylar cage for mating and oviposition. Egg masses laid on the same day were gathered and placed in plastic plates for holding and incubation. Neonates hatching on the same day were selected for culture maintenance and served as stock culture for morphological and biological characterization. Rearing was done at 27-28℃, RH 60-70%, and light/dark periods, 12L:12D.

Figure 1. Collection site of *Spodoptera frugiperda* in Barangay Annanuman, San Pablo, Isabela, Luzon, Philippines

Figure 2. (a) Monitoring *Spodoptera frugiperda* in rice; (b) larva feeding on rice leaf; (c) irregular holes on leaves of rice; (d) mature larva feeding on chewed-up tiller; (e) larva anchored on a fallen leaf of rice on water ; (f) and seedlings; (g) mature larvae collected during seedling pulling (Photo credits: Minda Flor Aquino)

Morphological examination and identification

Field-collected larvae were identified morphologically based on larval, pupal, and adult materials already on hand (Navasero and Navasero, 2020) and through the use of online services (e.g. CABI, 2018). The majority of the larvae were reared to adulthood and served as parental stock. Observations on egg and early larval instars of progenies were done under laboratory conditions.

Strain identification through COI gene fragment

The total genomic DNA of ten individual field-collected larvae was isolated from the thoracic muscles using the Promega Wizard® Genomic DNA Purification Kit (Promega, Madison WI). The *COI*A region was amplified using JM76 and JM77 primers following the method of Nagoshi *et al.* (2018). The target amplicons were evaluated through electrophoresis resolved in 1.2% agarose gel stained with GelRed® (www.biotium.com) and visualized under 302-nm UV light using AlphaImager® MINI (ProteinSimple, San Jose, California, USA). Amplicons were sent for sequencing at Apical Scientific Sdn. Bhd. (Taman Serdang Perdana, 43300 Seri Kembangan, Selangor, Malaysia) through AsiaGel Corporation (Villa Lourdes Townhomes, Congressional Avenue, Barangay Bahay Toro, Quezon City, Philippines). Sequences were processed using BioEdit (Hall, 2011) then subjected to the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi, 2022) for

species confirmation. Sequences generated from this study were submitted to GenBank (Accesion Numbers ON797720-ON797729).

Life history study

Adult males and females were caged in pairs in a cylindrical Mylar plastic oviposition cage. Ten pairs were made in this way. The cylinder was covered top, bottom and sides with pieces of wax paper as egg laying substrate. Cotton wool soaked in 10% sugar solution was provided as a food source for the couples. From the progeny of these parents, 200 newborn larvae were individually transferred to fresh maize leaves (IPB var. 6) and reared in plastic dishes until pupation. Thirty individuals were closely observed for molting up until pupation. The morphological characteristics of the different stages were observed and recorded along with developmental periods from the first instar to the sixth instar, prepupal and pupal periods. Pictures of eggs, first-instar larvae and second-instar larvae were taken with a stereoscope (Carl Zeiss Steno 305) with built-in camera and Labscope version 2.81 software. All other stages were shot with a Canon camera. The remaining larvae were similarly fed and housed until pupation, sexed and individually weighed. The pupae were grouped by sex and weight. Heavy pupae weighed 254.18 mg for males and 257.25 mg for females and light pupae weighed 206.25 mg for males and 210.18 mg for females. The effect of pupal weight on female fertility (number of eggs laid during her lifespan) and on adult lifespan (time from adult hatching to death) was determined. All observation periods were taken in days. Egg hatch percentage was determined by counting the number of neonates hatched from all eggs laid by a female in her lifetime for both weight groups.

Results

Morphological identification

Field-collected larvae from rice seed beds in Barangay Annanuman, San Pablo, Isabela were mostly full grown, dark brown, and with granulated cuticle (Figure 3a) and typical white inverted “Y” on the head (Figure 3b). The dorsal pinacula on the 8th abdominal segment of the larva were arranged in a square and the pinacula on the 9th segment were arranged in a trapezoid (Figure 3c), typical of *S. frugiperda*. The resulting pupae were reddish brown in color, with two spines in the cremaster. Emerged adults exhibited sexual dimorphism typical of *S. frugiperda*. Male adults were greyish brown. Their forewings are light brown, with oval or oblique orbital spots, less contrasting transverse lines,

indistinct reniform spot faintly outlined in black, and white patch markings near the apical margin. The female adults, on the other hand, lacked distinct markings on their forewings which are uniformly greyish brown to rust brown and darker than the forewings of the males. The hindwings in both sexes were silvery with brownish apical borders.

Figure 3. Full grown larva of *Spodoptera frugiperda* showing (a) granulated cuticle, (b) white inverted Y head, and (c) pinacula on 8th abdominal segment arranged in a square and on 9th abdominal segment arranged in a trapezoid (Photo credits: M.V. Navasero)

Progenies from field-collected FAW were described as follows:

Egg: The egg was dome-shaped and dorso-ventrally flattened. Egg laying occurred on the inner side and on the upper and netter surfaces of the leaf in a mass deposited in layers (Figure 4).

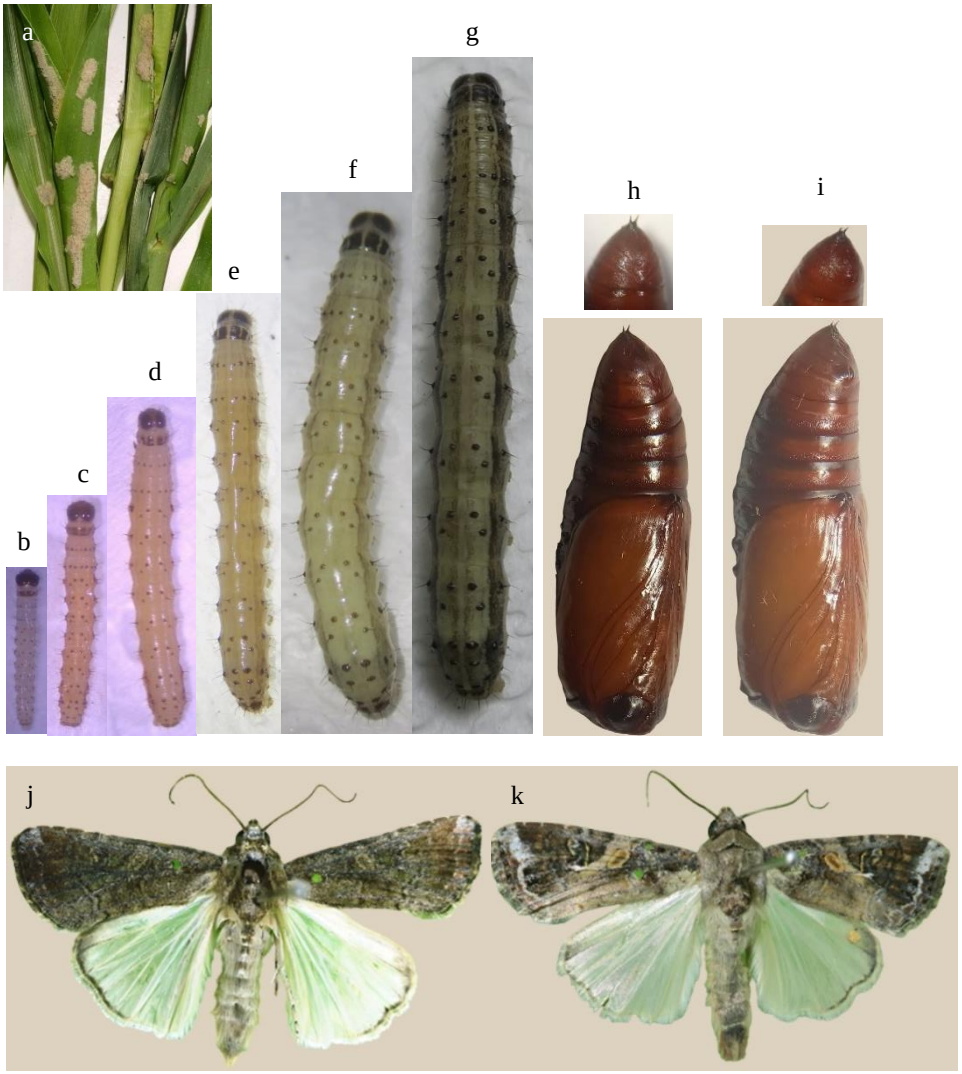

Figure 4. Progenies of *Spodoptera frugiperda* collected in rice: a.) egg clusters, b – g.) 1st to 6th instar larvae, h.) female pupa, i.) male pupa, j.) adult female, and k.) adult male

Larval stages: First and second instar larvae were greenish in color with black head. The final instars were dull gray in body color, with a typical inverted "Y" at the head capsule and distinct pinacula on the body. The pinacula were arranged in a square on the 8th segment and in a trapezoid from the first to the 7th segment and on the 9th segment.

Pupae: The pupae were reddish brown in color and the pupation under laboratory conditions was in between the tissue paper and leaves inside the rearing plate.

Adult: The male's forewings were grey-brown to rust-brown with a conspicuous triangular white spot at the apical regions, a faint renal spot bordered with black, a light brown, oval and oblique round spot, and a series of small markings near the apical regions. The female, on the other hand, had forewings that were uniformly greyish-brown to rusty brown and darker than the males. The hindwings are silvery with brownish apical margins in both sexes.

Strain identification using COIA gene region

Comparison of all the amplicon nucleotide sequences revealed a 100% identity match with the *S. frugiperda COI*A gene region with GenBank Accession Number MN599980.1 or MT732476.1 (Table 1). Alignment of the obtained nucleotide sequences showed polymorphisms within the *COI*A of *S. frugiperda* samples from Annanuman, San Pablo, Isabela, Luzon, Philippines (Table 2), revealing the presence of the two strains of FAW. These polymorphisms could distinguish *S. frugiperda* CS from RS. Indeed, further analysis of *COI*A region using DNA Sequence Polymorphism reveals the occurrence of two haplotypes in the nucleotide sequences obtained from the 10 samples, namely *COI*-RS and *COI*-CS, with 60% and 40% frequency, respectively.

Biology on Corn

Spodoptera frugiperda progressed from the egg through six larval and pupal stages before becoming adult (Table 3). The mean developmental time of the first, second, third, fourth and sixth instar larvae of both sexes was similar, while the fifth instar and pupal instar were not. The total larval period was slightly longer and the pupal duration was significantly longer in males than females. Therefore, the females hatched earlier than the males. However, the total development time of 20.61 days in males was slightly longer than the 18.85 days in females. However, the difference was not statistically significant.

Lifetime fecundity of females emerged from heavy pupae was higher (x = 1,857.75±858.07) than those of females emerged from light pupae (x = 1,390±303.03), but the difference was not statistically significant (Table 4). Percent egg hatch was also higher among heavy pupae (x = 74.15±23.16) than among light pupae. However, longevity was significantly longer in male adults emerged from light pupae. (x = 19.17±6.87 days) than from heavy pupae (x = 9.27±3.9 days). A similar trend was observed among the female adults (x = 19.29±7.41 days versus 11.9±2.47 days).

Table 1. Summary of the most significant BLAST hit of *cytochrome C oxidase I* gene region of *Spodoptera frugiperda* collected in rice in seedbeds in Annanuman, San Pablo, Isabela, Luzon, Philippines with the reported mitogenome of *S. frugiperda* in the GenBank

S. frugiperda **Query Nucleotide Sequence**	**Subject Sequence**				
	Description	**E Value**	**% Identity**	**Authors/ Country**	**GenBank Accession Number**
ON797720 ON797721 ON797722 ON797725 ON797727 ON797728	*Spodoptera frugiperda* mitochondrion, complete genome (1461 to 2991 gene region)	0	100%	Kim *et al.*, 2020 Republic of Korea	MN599980.1
ON797723 ON797724 ON797726 ON797729	*Spodoptera frugiperda* mitochondrion, complete genome (1463 to 2993 gene region)	0	100%	Liu, 2021 China	MT732476.1

Table 2. Summary of the polymorphic sites and the corresponding nucleotides of the *cytochrome C oxidase I* gene region of rice strain (*COI*-RS) and corn strain (*COI*-CS) of *Spodoptera frugiperda* collected in rice in seedbeds in Annanuman, San Pablo, Isabela, Luzon, Philippines

Haplotype No. (N) GenBank Accession Number	**Polymorphic Sites**									
	60	**117**	**198**	**237**	**249**	**255**	**270**	**289**	**360**	**459**
***COI*-RS (6)** ON797720 ON797721 ON797722 ON797725 ON797727 ON797728	C	G	C	T	C	T	A	A	A	T
***COI*-CS (4)** ON797723 ON797724 ON797726 ON797729	T	A	T	G	T	C	G	T	G	C

Table 3. Duration (in days) of various developmental stages of male and female *Spodoptera frugiperda* fed corn leaf from parental stocks collected in rice in Isabela, Philippines under laboratory conditions. Means for each parameter followed by a common letter are not significantly different for the comparison between treatments within each column (t-test, P<0.05)

Development Stage	Sex						T- test	
	Male			Female				
	Mean (day)	±	SD	Mean (day)	±	SD	*P-value*	t_c, P<0.05
Egg	2.00			2.00				
Larva								
First instar	1.61	±	0.21[a]	1.58	±	0.19[a]	0.6999	2.0595
Second instar	1.25	±	0.26[a]	1.27	±	0.26[a]	0.8489	2.0595
Third instar	1.50	±	0.55[a]	1.27	±	0.56[a]	0.2939	2.0595
Fourth instar	1.25	±	0.26[a]	1.19	±	0.25[a]	0.5644	2.0595
Fifth instar	1.50	±	0.48[a]	1.08	±	0.19[b]	0.0065	2.0595
Sixth instar	1.96	±	0.31[a]	2.12	±	0.42[a]	0.2912	2.0595
Pre-Pupa	1.11	±	0.29[a]	1.12	±	0.22[a]	0.9346	2.0595
Total larval period	10.18	±	0.99[a]	9.62	±	0.98[a]	0.1512	2.0595
Pupa*	8.43	±	0.51[a]	7.23	±	0.60[b]	< 0.0001	2.0595
Total development period (Egg to Adult)	20.61	±	1.00[a]	18.85	±	1.26[b]	0.0005	2.0595

Table 4. Weight of pupa, fecundity, hatchability of eggs, and longevity of *Spodoptera frugiperda*

Parameter		Sex									
		Male					Female			T- test	
		Mean	±	SD	*P-value*	t_c, P<0.05	Mean	±	SD	*P-value*	t_c, P<0.05
Weight (mg) Pupa	Heavy	254.18	±	4.12[a]	< 0.0001	2.0796	257.70	±	8.10[a]	< 0.0001	2.0595
	Light	206.25	±	17.47[b]			210.18	±	20.57[b]		
Fecundity	Heavy ♀ Heavy ♂						1857.75	±	858.07[a]	0.2293	2.3060
	Light ♀ Light ♂						1390.0	±	303.03[a]		
Percent egg hatch	Heavy ♀ Heavy ♂						74.15	±	23.16[a]	0.5225	2.3060
	Light ♀ Light ♂						66.02	±	21.82[a]		
Longevity, days	Heavy	9.27	±	3.90a	0.0004	2.0796	11.90	±	2.4[7a]	0.0055	2.0106
	Light	19.17	±	6.87b			19.29	±	7.41[b]		

Means for each parameter followed by a common letter are not significantly different for the comparison between treatments within each column (t-test, P<0.05).

Discussion

Based on the morphological descriptions of *S. frugiperda* larvae by Visser (2017); Srikanth *et al.* (2018); Navasero *et al*. (2019), and Navasero and Navasero (2020), the larvae collected from rice in this study were identical to *S. frugiperda.* Adult *S. frugiperda* emerged from the same colony showed the same coloration of both sexes described by Visser (2017), Shylesha *et al.* (2018), Navasero *et al*. (2019), and Navasero and Navasero (2020). The male and female wingspans documented in the study were also within the range reported by Navasero and Navasero (2020).

Reports showed that *S. frugiperda* is polyphagous, infesting rice (Pashley, 1986), beans (Pogue, 2002), cotton (Hardke *et al.*, 2015), sorghum (Venkateswarlu *et al.,* 2018), soybean (Thrash *et al.,* 2013), sugarcane (Kasambala Donga and Meadow, 2018), potato, and peanut (Amusan, 2018). Montezano *et al.* (2018) reported 353 host plants for FAW. In the Philippines, rice, corn, cotton, sorghum, sugarcane, beans, and vegetables are typically planted, allowing movement of FAW between crops.

The *COI*A region is utilized in confirming the species and strain haplotypes of *S. frugiperda* (Nagoshi *et al.,* 2018). The presence of two strains of FAW in the population from rice is an indication of polymorphism in *S. frugiperda* (Nagoshi *et al.,* 2007), distinguishing CS from RS. Further analysis of *COI*A region using DNA Sequence Polymorphism (Librado and Rozas, 2009) confirmed the occurrence of two haplotypes: *COI*-CS and *COI*-RS. The RS was originally detected in corn on June 7, 2019 from samples (IPPC, 2019) from Piat, Cagayan and Isabela which are corn-growing areas in the Philippines.

In the present study, 40% of the colony was RS and 60% CS. Although RS preferred rice as a host plant, it can also be found in other plants such as pasture grasses (Nagoshi and Meagher, 2003 a and b). Both CS and RS have been observed in corn and other crops in America (Unbehend *et al.,* 2014) and in Africa (Jacobs *et al.,* 2018). CS has also been collected in cotton (Nagoshi *et al.,* 2007) and sorghum (Venkateswarlu *et al.,* 2018). In the biological studies of *S. frugiperda* on young corn leaves (IPB var 6), the mean developmental time of larvae at the first, second, third, fourth, and sixth instar larvae of both sexes had similar durations, while the fifth instar and pupa stages were not. The total larval period was slightly longer and the pupal duration was significantly longer in males than females. Therefore, the females hatched earlier than the males. A significantly longer total development time (20.61 days) was recorded in males than in females (18.85 days). The results were shorter than previous reports by Navasero and Navasero (2020). Sharanabasappa *et al.* (2018) reported that the full life cycle of FAW males and females, including adult

longevity, was 32-43 days and 34-46 days, respectively. An average complete developmental cycle of 39.79±4.59 days was reported by Nguessan *et al.* (2021).

The present study showed that heavy pupae generally produce larger adults that lay more eggs. However, the difference between the fecundity observed in adults emerged from light and heavy pupae was not statistically significant. Still, the results were within the range of 1,061 to 1,850 eggs per female reported by other workers (Bernardi *et al.*, 2014; Pinto *et al.*, 2019). The longevity of males and females in this study was within the range established by Sharanabasappa *et al.* (2018), Pinto *et al.* (2019), and Navasero and Navasero (2020).

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