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THE RED PALM WEEVIL IN THE UAE: MORPHOLOGICAL DIVERSITY AND RNAI-MEDIATED GENE SILENCING OF TWO CUTICLE-RELATED GENES

Safa Hashem Mohammed Musaed

United Arab Emirates University

College of Science

Department of Biology

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Safa Hashem Mohammed Musaed

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Environmental Sciences

Under the Supervision of Dr. Mahammad Ali Al-Deeb

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Declaration of Original Work

I, Safa Hashem Mohammed Musaed, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*The Red Palm Weevil in the UAE: Morphological Diversity and RNAi-Mediated Gene Silencing of Two Cuticle-related Genes*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Mohammad Ali Al-Deeb in the College of Science at UAEU. This work has not previously been presented or published or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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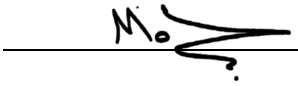
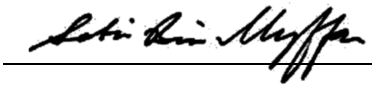
Approval of the Master Thesis

This Master Thesis is approved by the following Examining Committee Members:

1) Advisor: Dr. Mohammad Ali Al-Deeb
   Title: Associate Professor
   Department of Biology
   College of Science
   Signature  Date 12-Dec-2021

2) Member: Prof. Sabir Bin Muzaffar
   Title: Professor
   Department of Biology
   College of Science
   Signature  Date 12-Dec-2021

3) Member: Dr. Bridget Howarth
   Title: Associate Professor, Director of Institutional Effectiveness
   Institution: American University in Dubai, UAE
   Signature  Date 14-Dec-2021

This Master Thesis is accepted by:

Dean of the College of Science: Professor Maamar Benkraouda

Signature maamar Benkraouda Date Jan. 4, 2022

Dean of the College of Graduate Studies: Professor Ali Al-Marzouqi

Signature Ali Hassan Date Jan. 4, 2022

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Abstract

Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae), threatens palm trees worldwide. A better understanding of this insect can help with designing an adequate management strategy. This study aimed to a better understand the morphological diversity of RPW and examined RNAi-mediated gene silencing of two cuticle-related genes, vestigial (vg) gene, and Laccase (Lac2) gene by injecting the last larval stage with double-stranded RNA (dsRNA). For the morphological diversity study, adults of RPW were collected and classified by their prothoracic spots. Additional morphological characters were measured such as Pronotum Length (PL), Pronotum Width (PW), Elytra Length (EL), Elytra Width (EW), and total Length (L), as well as observing the density of the hair-like structure on the male rostrum. The data were analyzed using descriptive statistics, scatterplots to present data distribution within the typologies, box-and-whisker plots to show the distribution of the body length, Student *t*-test conducted to compare the body length (TL) between typologies, and percentage to reflect hair-like structure density. For RNAi experiments, total RNA was extracted and double-stranded RNA (dsRNA) was prepared to inject the RPW larva. For vg gene, two doses were used (1,800 ng and 5,600 ng), and 5,600 ng for Lac2 gene. To measure the expression level, quantitative Real-Time Polymerase Chain Reaction (RT-qPCR) was performed. The morphological study showed the presence of seven prothoracic spot typologies and addressed the morphological differences and the three levels of rostral setae on the male's rostrum. Besides that, dsRNA had successfully silenced the vg and lac2 genes in *R. ferrugineus*, resulting in adults emerging with developmental abnormalities that can affect the insect's survival and reproduction.

Keywords: *Rhynchophorus ferrugineus,* morphological diversity, typology, RNA interference (RNAi), double-stranded RNA (dsRNA), quantitative Real Time - Polymerase Chain Reaction (RT-PCR).

Title and Abstract (in Arabic)

سوسة النخيل الحمراء في الإمارات العربية المتحدة: التنوع المورفولوجي والإسكات الجيني بواسطة RNAi لجينين مرتبطين بالبشرة

الملخص

تهدد سوسة النخيل الحمراء أشجار النخيل في جميع أنحاء العالم. يمكن أن يوفر لنا الفهم الأفضل لهذه الحشرة استراتيجية إدارة مناسبة. هدفت هذه الدراسة إلى فهم أفضل للتنوع المورفولوجي لسوسة النخيل الحمراء واختبار إسكات الجينات بوساطة تدخل الحمض النووي الريبي للجينات المرتبطة بالبشرة وهما جين vestigial (vg) وجين Laccase (Lac2) عن طريق حقن (dsRNA) في المرحلة الأخيرة من الطور اليرقي. لدراسة التنوع المورفولوجي، تم جمع سوسة النخيل الحمراء البالغة وتصنيفها حسب أنماط البقع الداكنة في المنطقة الصدرية. كما تم قياس بعض الصفات المورفولوجية مثل طول القصبة (PL)، وعرضها (PW)، وطول جناح الإليترا (EL)، وعرضها (EW)، والطول الكلي بدون الخطم (L)، وكثافة الشعر في خطم الذكور. تم تحليل البيانات باستخدام الإحصائيات الوصفية، واستخدام مخطط (scatterplots) لعرض توزيع البيانات ضمن الأنماط، ومخطط (box-and-whisker) لإظهار توزيع طول الجسم، وتم إجراء (Student t-test) لمقارنة طول الجسم (L) بين الأنماط. أما بالنسبة إلى تدخل الحمض النووي الريبي، تم استخلاص RNA من بالغات سوسة النخيل الحمراء من ثم تحضير الحمض النووي الريبي مزدوج الشريط (dsRNA) وحقن يرقات الطور الأخيرة. حددت دراسة مورفولوجية الحشرة سبع أنماط للبقع في المنطقة الصدرية وتناولت الاختلافات المورفولوجية، وأوضحت المستويات الثلاثة من الهياكل الشبيهة بالشعر في خطم الذكر. إلى جانب ذلك، نجح الحمض النووي الريبي في الإسكات الجيني لجينيّ (Lac2،vg) في سوسة النخيل الحمراء، مما أدى إلى ظهور تشوهات في النمو البالغات والتي يمكن أن تؤثر على بقاء الحشرة وتكاثرها.

مفاهيم البحث الرئيسية: سوسة النخيل الحمراء، التنوع المورفولوجي، التصنيف، التعبير الجيني، الحمض النووي الريبي مزدوج الشريطة، الوقت الحقيقي الكمي - تفاعل البلمرة المتسلسل.

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Dedication

To my beloved parents and brothers

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List of Abbreviations

dsRNA	Double-strand RNA
EL	Elytra Length
EW	Elytra Width
GFP	Green Fluorescent Protein
Lac	Laccase Gene
PL	Pronotum Length
PW	Pronotum Width
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
RNAi	RNA interference
RPW	Red Palm Weevil
TL	Total length
UAE	United Arab Emirates
vg	Vestigial Gene

Chapter 1: Introduction

1.1 Overview

The Date palm (*Phoenix dactilyfera*) is a significant source of fruit and an ornamental tree worldwide. It belongs to the family, *Arecaceae* (Palmaceae), which has more than 200 genera and over 2,500 species (Corner, 1966). Palm are a critical crop in many countries, as their fruit is considered the primary food for these countries. According to Food and Agriculture Organization (FAO) statistics, United Arab Emirates (UAE) considered the second country in the Gulf region in producing the date palm reaching 318 thousand tons in 2001 (Botes & Zaid, 1999). This produced amount has a threat with a pest associated with the date palm, and one of these pests is the red palm weevil. Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* (Oliver) (Coleoptera: Curculionidae), is known as one of the most dangerous pests on different palm species around the world (Abraham et al., 1998; Faleiro, 2006). The most known integrated pest control methods used to control RPW were insecticides and pheromone-based traps. Besides these methods, many technologies have aided in controlling it, such as sensitive microphones that can detect larvae feeding inside a tree, drones, and remote sensing. Annually, about $8 million is lost due to removing severely infested trees in Gulf countries and the Middle East (FAO, 2018).

Over the years, insect resistance has risen against the insecticides; the chemical control methods were the most straightforward way farmers practiced, which have adverse effects on the environment and cause toxicities for vertebrates and invertebrates. (As mentioned earlier), All the effects could affect insects' genetic diversity, which makes it necessary to develop novel and sustainable approaches to

control agricultural pests (Rodrigues & Figueira, 2016). RNA interference (RNAi) is defined as a process when a post-transcriptional gene expression inhibition triggered by small interfering RNA (siRNA) intercede to silence the gene by destroying the homologous mRNA fragment (Hannon, 2002). The gene silencing method mediated by double-stranded RNA (dsRNA) was discovered in 1990 in petunias (Napoli et al., 1990). Then it was explained in the nematode *Caenorhabditis elegans* when Fire and his colleagues want to investigate the gene expression, thus, it helped in considering the RNAi as one of the strategies in controlling the insect pests (Fire et al., 1998).

Using RNAi in controlling insects is known as RNAi-mediated crop protection. Since the RNAi responses can be obtained in all species, the use of RNAi as a tool to examine the function of essential genes has increased dramatically, which had a higher throughput RNAi screening tools (Hannon, 2002; Kuttenkeuler & Boutros, 2004).

1.2 Statement of the Problem

From the mid-1980s, *R. ferrugineus* is considered as a threat to date palm production in Gulf Cooperation Council (GCC) countries, and several methods were involved to control this destructive pest. Chemical insecticides were the primary strategy until the aggregation pheromone was synthesized. In the UAE, no RNAi studies have been conducted on any agricultural pest so far. In addtion, no morphological study was done covering the difference in body dimensions and spot typologies. The current study is a step towards reaching a deeper understanding of this major invasive pest and providing a precursor to a non-chemical control method. At the same time, In the UAE, morphological diversity and prothoracic spot differences of *R. ferrugineus* needs to be studied. This study is vital to complement the lack of research using RNAi in the UAE.

1.3 Relevant Literature

1.3.1 Date palm

Date palm (*P. dactilyfera*) is an essential fruit source worldwide. It belongs to the *Arecaceae* (Palmaceae) family, which has more than 200 genera and more than 2,500 species such as *P. canariensis* (Canary Island palm), *P. reclinate* (Senegal date palm), and *P. sylvestris* (Indian sugar date palm) (Corner, 1966). According to Jain et al. (2009), the date palm is considered the tallest comparing with all the *Phoenix* species; the trunk can grow under some conditions to reach 30 m. It is classified as a dioecious species, where it is known that the male and female organs are carried on a separate tree.

1.3.1.1 Date Palm History

According to Jain et al. (2009), the date palm is an ancient plant that people have used for a very long time. Many studies show the difference in the date palm's origin, where the representations of this plant appear in hieroglyphic engravings in old Egypt. Some Akkadian and Sumerian cuneiform sources recorded the date palm in the old history in the following areas extending from Indus Valley (Pakistan) to Tigris/Euphrates valleys (Iraq), the Nile valley, Southern Persia to the Eastern Mediterranean. This indicates that the first cultivations for date palm were about 4000 B.C. from the coast of the Persian Gulf and Iraq. Besides, Jain et al. (2009) mentioned that the oldest discovery of date palm seeds was on Dalma island, one of the Abu Dhabi Islands. In 1998, they found two seeds and these were dated from 5110 B.C. and 4670 B.C, which mean that the record of date palm in the Islamic world was from 5000 B.C.

1.3.1.2 Date Palm Ecology

As the origin of date palm is believed to be from Pakistan to Iraq, the climate in those regions is dry and sunny most of the year. The date palm can tolerate areas of scarce rain, dry and abundant sunshine with maximum temperatures of around 50^{o}C. Also, it can tolerate very cold and wet conditions (Saudi Aramco World, 1962; Barreveld, 1993).

1.3.1.3 Date Palm Importance

The date palm has religious, social, medical, and commercial value. It is associated with the human religiously, where it is mentioned in the Qur'an and Bible with many replications (Jain et al., 2009). In many countries, dates are one of the national heritage fruit crops, especially in the Gulf region where the climate is very suitable to grow it (El-Juhany, 2010). Dates have nutritional value; it contains a high concentration of protein, vitamins, and mineral salts (Barreveld, 1993). Also, the date palm was used for many commercial uses; the disgarded material resulting from annual pruning of palm trees can be used as a construction material for different purposes such as roofs, fences, baskets, and textiles, which results in zero waste from the growing of palms (González-Marín et al., 2012; Barreveld, 1993) (Figure 1).

Figure 1: Homemade material done by knitting palm tree fibers and leaves (Manachini et al., 2013)

Medically, different parts of palm trees are used to treat many diseases. The date fruits contain compounds known to possess multiple beneficial effects (Mahmoudi et al., 2008). El-Juhany (2010) mentioned that dates were used with other herbs to treat the digestive system organ, because of their tannin content. Also, palm fronds are applied as medication for nerviness and tension, kidney, and blood problems. Toothaches can be treated by using the date's root (Abdullahi & Garko, 2012)

1.3.1.4 Date Palm Insect Pests

Many agricultural pests and diseases are associated with the date palm. Over 100 insects and mites can infest the palms, which can be distributed among eight insect orders and one mite order. Each part of the palm hosts a different type of pest. Although the number of date palm tree pests is high, just a few are considered of significant economic importance. The significant pests include the red palm weevil

(*Rhynchophorus ferrugineus*), lesser date moth (*Batrachedra amydraula*), Dubas date bug (*Ommatissus lybicus*), green pit scale (*Palmaspis phoenicis*), carob moth (*Ectomyelois ceratoniae*), date palm longhorn beetle (*Jebusaea hammerschmidti*), almond moth (*Cadra cautella*), and old-world date mite (*Oligonychus afrasiaticus*) (El-Shafie et al., 2017).

1.3.2 Red Palm Weevil

Rhynchophorus ferrugineus (Oliver) (Coleoptera: Curculionidae), which is known as the Red Palm Weevil (RPW), is a very destructive insect pest. It invaded the Gulf countries in the mid 1980s and from that time it has economic importance in many parts of the Gulf (Al-Dosary et al., 2016).

1.3.2.1 Scientific Classification

Weevil borers of palms are members of seven natural lineages within the 'Curculionidae', and it belongs to the Dryophthoridae which is being the most damaging to palms worldwide. Rhynchophorini tribe includes the genera Dynamis and Rhynchophorus (Giblin-Davis, 2001; Giblin-Davis et al., 2013). The taxonomy of red palm weevil is shown as it was described by Harpootlian et al. (2011) and is given below:

- Kingdom: Animalia (Animals).
- Phylum: Arthropoda (Arthropods).
- Subphylum: Hexapoda (Hexapods).
- Class: Insecta (Insects).
- Order: Coleoptera (Beetles).
- Suborder: Polyphaga (Water, Rove, Scarab, Long-horned, Leaf and Snout Beetles).
- No Taxon: (Series Cucujiformia).

- Superfamily: Curculionoidea (Snout and Bark Beetles).
- Family: Curculionidae (Snout and Bark Beetles).
- Subfamily: Dryophthorinae.
- Tribe: Rhynchophorini.
- Genus: *Rhynchophorus.*
- Species: *ferrugineus* (Red Palm Weevil).

1.3.2.2 Red Palm Weevil Distribution

Rhynchophorus ferrugineus (RPW) is found over a very wide geographical area, involving many different climates. It has been distributed worldwide except in Antarctica (Figure 2). The first observation of RPW was overlapped with two palm species, *Cocos nucifera*, in South and Southeast Asia and *P. dactylifera* in the Middle East (Fiaboe et al., 2012). According to Viado and Bigornia (1949), southeast Asia is considered the homeland of the RPW in the coconut palms. In 1918, the first record of infested palm was in India and Punjab's southern regions (Milne, 1918). This weevil spread widely during the last three decades due to palm trees' commercial activities of human intervention by transporting infested date palm trees and offshoots from the Middle East to Africa, the Mediterranean area, and other regions (Ferry & Gomez, 2002; Dembilio & Jaques, 2015). These activities result in reporting the first record in Europe in Spain (Cox, 1993; Barranco et al., 1995). According to El-Ezaby et al., (1998), the first report of RPW in the UAE was in 1985. As spreading continued, Murphy and Briscoe (1999) mentioned, RPW was found in Saudi Arabia in 1987 and in Iran in 1992. North Africa had tried to avoid the RPW until December 2008, when it was reported in the Tangier region in Northern Morocco. Since then, it became the most important insect pest on date palm in UAE because the damage is often severe when it is discovered (Abraham et al., 1998).

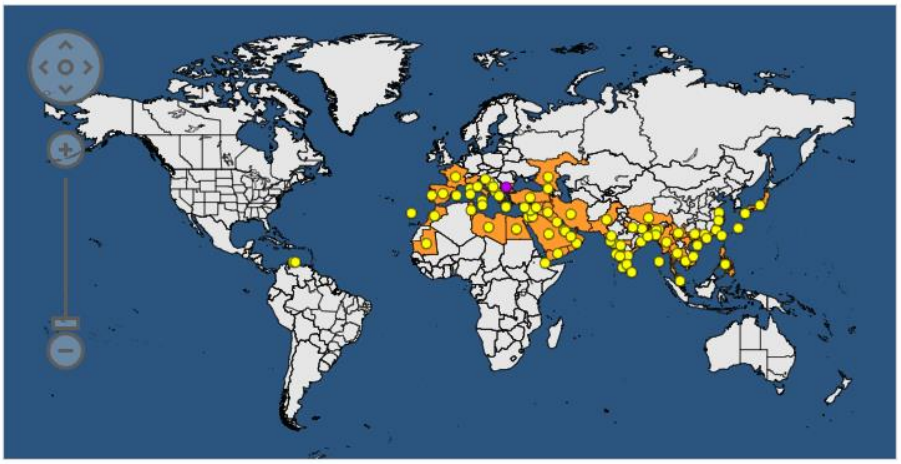

Figure 2: The Distribution of Red Palm Weevil in the World (EPPO, 2020)

1.3.2.3 Insect Life Cycle

Rhynchophorus ferrugineus is holometabolous, which means that it has a complete life cycle, from the egg to a mature adult (Figure 3).

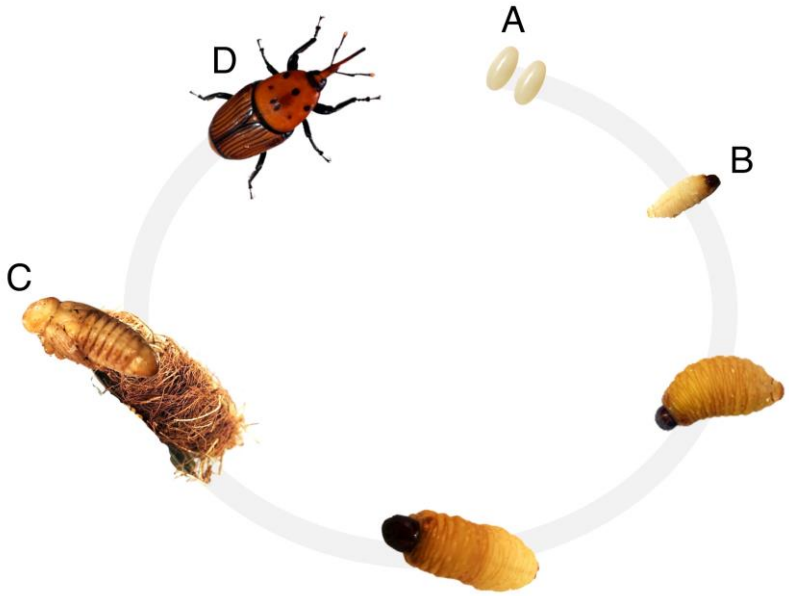

Figure 3: *R. ferrugineus* Life cycle. Eggs: (A) creamy white eggs, (B) Larva: A complete larva has a yellowish-white body and a reddish-brown head with strong mandibles, (C) Pupa: It starts with creamy color, then it turns to brown, with shiny surface. (D) Adult: Reddish brown, with a long-curved rostrum; dark spots on the upper side of thorax.

Female weevils lay their eggs individually in separate holes excavated by the rostrum at the fronds' base. Eggs are yellowish white in color with a measure of 2.5 mm long, and they mainly hatch after 3 to 6 days (Kubar et al., 2017; Murphy & Briscoe, 1999) (Figure 3 A). Larvae, which is the damaging stage of the *Rhynchophorus* species, have whitish-yellow body with a brown rust-red head capsule and strongly chitinous mouthparts. They are legless, and their body is comprised of 13 segments (Figure 3 B). Depending on the diet, host plant, and temperature many studies showed a fluctuated variation in the larvae development period and the number of larval instars (Dembilio & Jacas, 2011, 2015). Martín-Molina and Cabello (2004) had studied larvae development in three different mediums, sugarcane lumps, palm lumps, and artificial diet; each one of them showed a relatively similar total larval instar, 11–17, 8–15, and 7–12, respectively. Abe et al. (2009) mentioned that larvae had 10 to 13 instars during their life in apple slices medium. Dembilio and Jacas (2011) reported that RPW larvae completed a total of 13 instars in live P. *canariensis* palms. However, Salama and Saker (2002) estimated that it had 21 generations annually in Egypt.

Larvae feeding in different palm tissues according to their age, where the soft tissues around the apical meristems are favorable for the young larvae. In contrast, mature larvae move to the trunk's core or to the crown to help it convert to the pupal stage covered with palm fibers (Wakil et al., 2015). Generally, the pupation occurs in the base of palm’s frond. The pupa is creamy in color, and then turns pale brown with an average size of 80x35 mm (Murphy & Briscoe, 1999) (Figure 3 C). The pupal stage's life range varies from 11–45 days (Viado & Bigornia, 1949; Esteban-Durán et al., 1998; El-Zoghby & Abdel-Hameid, 2018) and in UAE it had reported that the pupal period range between 23–26.5 days (El-Ezaby et al., 1998). Finally, the last stage

of the RPW is the adult. Adult RPW is large, about 35x10 mm long, rusty red with a characteristic long and curved rostrum, consisting of one-third of the total length (Figure 3 D). On the dorsal side of the thorax, the weevils exhibit dark spots. In males, the anterior dorsal half of the rostrum has short brownish rostral setae (hairs structure-like). By contrast with female, rostrum lacks any setae, is narrower, curved, and longer than the male rostrum (Tambe et al., 2013). The adult weevils have well-developed wings and can undertake long flights, as mentioned by Lepesme and Ghesquiere (1947), and cover long distances of 500–800 m (Wattanapongsiri, 1965). RPW has two active generations, with more than two overlapping generations due to the length of the oviposition period. The first generation is considered the main and most economically important (Abdel-Wahed et al., 2014). This generation occurrs between March and early July, and the second active brood takes place at the end of September to the end of November, with a relatively moderate peak size at the end of October (Abdel-Wahed et al., 2014).

A field study done by Abdel-Wahed and his colleagues (2014), observing the location of RPW infestation on the trunk of date palm trees, showed that till 100 cm height of the trunk gets infested with 77.9% while 98.7% of infestation occurred up till 200 cm. According to the same study, 67% of infestation occurred in the second category of date palm trees, which concluded that the most preferred palm trees for the RPW aged between seven and ten years.

Sahito et al. (2017) tested the life cycle period's longevity on three different palm varieties (Aseel, Fasly and Karbalian). The minimum time needed for the RPW to complete the whole life cycle was almost the same, about 30 days on all three varieties, but there was a significant variation in maximum time. In Fasly variety, the life cycle was up to 100 days where it had completed up to 110 days on the Aseel

variety. The maximum life cycle duration was on the Karbalian variety, where RPW completed up to 120 days under laboratory conditions (Sahito et al., 2017).

1.3.2.4 Symptoms of Infestation

Rhynchophorus ferrugineus, insect borer, caused a lot of significant and economic damage losses to the date palms. Abraham and others (1998) had reported the symptoms of damaged palm tissue as the following the occurrence of the tunnels on the palm trunk and or on the base of frond petiole, thick brown fluid with a stinking smell with or without the presence of the ground tissues from the palm tunnels (Figure 4). Also, the presence of empty pupal cocoons and dead adults around a palm and dried offshoots appear. In the case of severe and prolonged infestation, falling of the crown or collapse of the palm trunk can easily occur.

According to El-Ezaby et al. (1998), infested palm trees are categorized on a scale of 0-5, where zero represents palms showing no damage symptoms or insect pest in any life stage, while five indicates the severest phase of attacks.

Figure 4: Red Palm Weevil symptoms in date palm orchards in Saudi Arabia (Abraham et al.,1998)

1.3.2.5 Host Range

Because the RPW is spreading in many countries, knowing the host species helps limit the infestation spreading. Host range of RPW, as reported by Nirula (1953), has significantly increased from just four palm species (*Cocos nucifera, Phoenix dactylifera, Metroxylon sagu, and Corypha umberaculifera*) in the mid-1950s. The number had increased to reach 18 host species listed by (Esteban-Durán et al., 1998).

1.3.2.6 Red Palm Weevil Damage and Treatment

The impact of invasive exotic pests is increasingly recognized as a global issue. In Italy, especially in Sicily, RPW caused the loss in more than 14,000 palms, which was mainly observed on *P. canariensis*, followed by *P. dactylifera* and *Washingtonia* spp. (Manachini et al., 2013).

According to FAO statistics in 2013, plant diseases and pests caused losses estimated at 30% in the global production of dates. In the Gulf region, the annual loss from the elimination of high-level infested palms has been estimated as US$1.74 at 1% of infestation, and it reaches around 8.69 million at 5% of infestation (Al-Dosary et al., 2016; El-Sabea et al., 2009).

Researchers have adopted Integrated Pest Management (IPM) approaches consisting of prohibiting infested plants' movement, use of pheromone traps, and planed insecticide use (El-Bokl et al., 2010). These strategies have been used to manage RPW in India (Murphy & Briscoe, 1999). The major components of the IPM were the visual inspection of the pest, plant, and field sanitation, using pheromones trap, applying chemicals (fungicide and insecticide) to the infected plants, adding to that, removing the severely infected trees, and educating farmers about pest management. However, these approaches were labor-intensive and expensive to

implement, which call for innovative management methods that utilize advanced technologies, including biological control, genetic engineering, and biotechnology. The combined implementation of these methods will ensure effective eradication of this weevil. These methods had proven the effectiveness of containing the pest's spread in Mauritania and the Canary Islands, where the pest has been successfully eradicated in May 2016 (Terry, 2017).

Researchers had modified the IPM programs to match the agroclimatic conditions of the Middle East. Introducing periodic surveys supports the detection of infestations using the symptoms mentioned above. In large areas, pheromone traps are used as monitors to enable the farmer to detect the weevil's presence (El-Bokl et al., 2010). The pheromone traps perform three distinct objectives: monitor the weevil activity, detect its presence, destroy the weevil population, and assess the weevil's population level (Abraham et al., 1998). In addition, new detection methods are used to detect the pest in the early stage of infestation. Many studies have been conducted on early detection of pest infestation; the most important detection method is related to larvae' vocal frequency/oscillation during their feeding period in the trunk (Gutiérrez et al., 2010; Mankin et al., 2011; Rach et al., 2013).

1.3.3 RNAi Technology

1.3.3.1 RNAi Overview

The phenomenon of RNA interference (RNAi) is a sequence-specific post-transcriptional gene regulation mechanism. This phenomenon occurs at a cellular level in response to the introduction of double-stranded RNA (dsRNA), which occurs widely among plants, animals, and microorganisms (Fire et al., 1998; Sen & Blau, 2006; Zhang et al., 2013). This technology allowed a unique progression in

understanding the gene function in organisms, therefore accelerating reverse genetics to new levels (Joga et al., 2016).

The first report of the RNAi phenomenon was in 1990 (Napoli et al., 1990). They tried to determine if the Chalcone Synthase (CHS) was the rate-limiting enzyme in anthocyanin biosynthesis responsible for the deep violet coloration in petunias. They overexpressed the chalcone synthase in petunias resulted in white petunias. They found that the endogenous and introduced CHS levels were lower than the wild-type petunias by 50-fold, so they hypothesize that the introduced transgene was "co-suppressing" the endogenous CHS gene. The same phenomenon was observed by Romano and Macino (1992) in *Neurospora crassa* when they noted that "quelling" of the endogenous gene is caused by the introduction of homologous RNA sequences.

The first RNA silencing experiment was documented in animals by Guo and his colleague Kemphues in 1995. They observed that the introduction of sense or antisense RNA to par-1mRNA resulted in degradation of the par-1 message in Nematoda named *Caenorhabditis elegans* (Guo & Kemphues, 1995). For more investigation, Fire and his colleagues conducted an experiment and reported the silencing of endogenous genes by "co-suppression, quelling and sense mRNA" in the nematode *C. elegans*, showing a paradoxical result to Guo and Kemphues (1995), where the ssRNAs was found less effective than dsRNA targeting the same mRNA. From this experiment, they established a new framework for the effects of RNA on gene silencing by highlighting a role for dsRNA, which helps consider the RNAi as one of the strategies in controlling the insect pests (Fire et al., 1998; Guo & Kemphues, 1995).

1.3.3.2 RNAi Mechanism in Insects

The three major characterized pathways of RNAi are the microRNA (miRNA), piwiRNA (piRNA), and small interfering RNA (siRNA) pathways. To apply RNAi technology in controlling insect pests by silencing a gene of interest, we have to activate the (siRNA) path by introducing the dsRNA into the insect body (Agrawal et al., 2003; Pecot et al., 2011). The pathway of RNAi in the cell was created by ribonuclease (RNase) III enzyme, this enzyme known as Dicer, which is used to shorten the dsRNA into small interfering RNAs (siRNAs) fragment (Elbashir et al., 2001) (Figure 5).

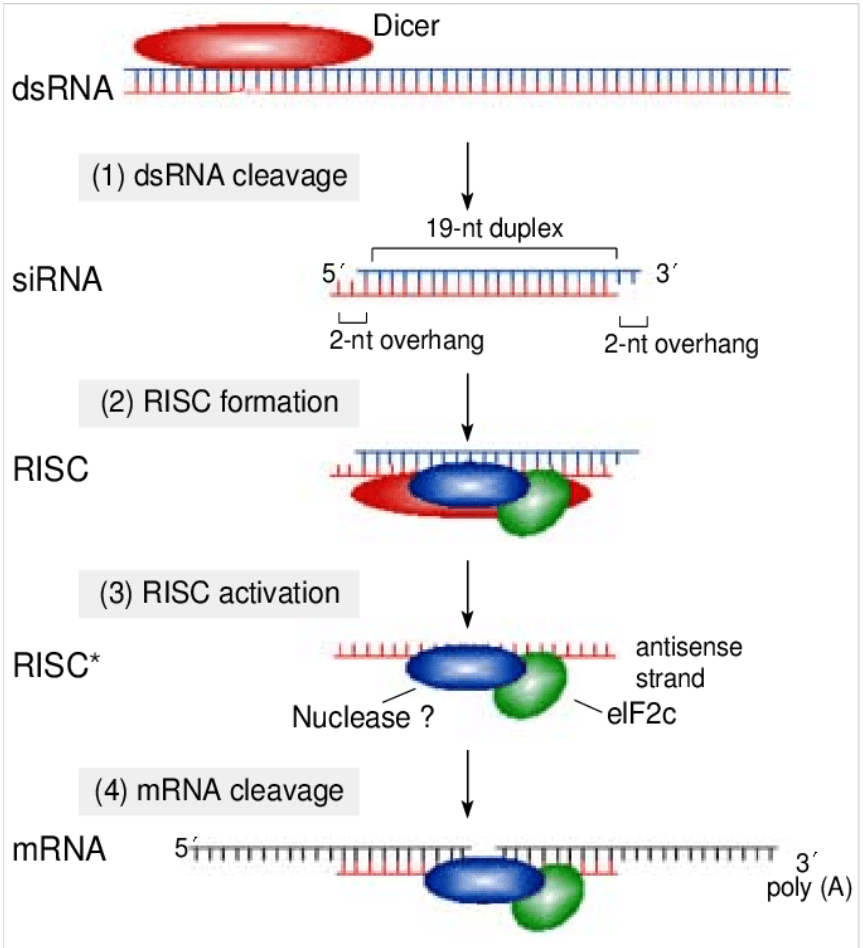

Figure 5: Cellular RNAi process in gene silencing (Kim, 2003)

The efficiency of delivering the RNAi is variable. The RNAi mechanism's effectiveness mainly depends on the delivery, stability, and uptake of dsRNA by target species (Huvenne & Smagghe, 2010). Moreover, Song et al. (2017) indicated that applying dsRNA through injection is more effective than oral delivery. The dsRNA uptake may have a different result depending on the availability of Sid-1 like genes (Cappelle et al., 2016; Xu et al., 2013). Moreover, many factors could affect the systemic silencing effect and the efficiency of RNAi as a tool in controlling insects. These factors are target genes, dsRNA design, dsRNA length, dsRNA concentration and insect tissue, life stage, and gut pH (Rodrigues & Figueira, 2016).

According to Whangbo and Hunter (2008), the use of the RNAi to control insect pests is part of the non-cell-autonomous (systemic and environmental) RNAi response. There are two mechanisms of the dsRNA up-taking in non-cell-autonomous RNAi. The environmental RNAi describes all processes in which dsRNA is taken up by a cell from the environment. In contrast, the systemic RNAi can only occur in multicellular organisms because it includes processes in which a silencing signal is transported from one cell to another or from one tissue type to another (Figure 6).

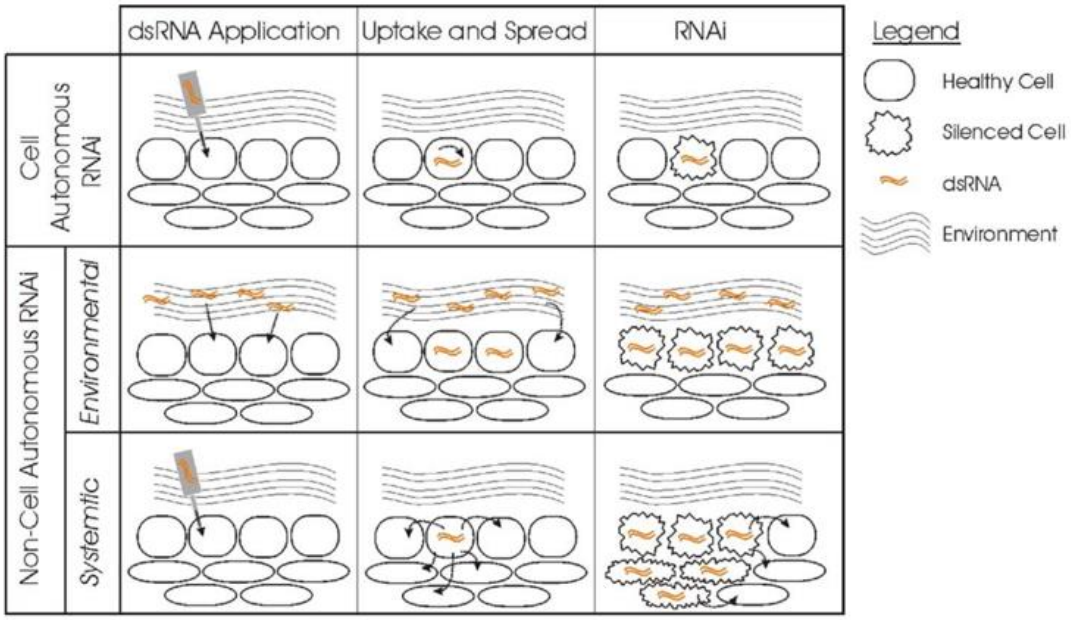

Figure 6: The difference RNAi response depending on the cell type (Whangbo & Hunter, 2008)

1.3.3.3 dsRNA Concentration

Sufficient gene silencing is affected by the concentration of dsRNA delivered to the insect; exceeding the concentration will not result in more silencing (Meyering-Vos & Müller, 2007; Shakesby et al., 2009). In contrast, it might decrease the duration of dsRNA exposure to reach 50% mortality (Bolognesi et al., 2012).

1.3.3.4 RNAi Delivery Methods

The success of RNAi technology is dependent on the dsRNA delivery strategies. The right selection of each insect's delivery method is necessary since the gene silencing is limited to the infected cell (Terenius et al., 2011). The most popular delivery methods are injection, feeding, soaking, and transgenic plants expressing dsRNA (Figure 7).

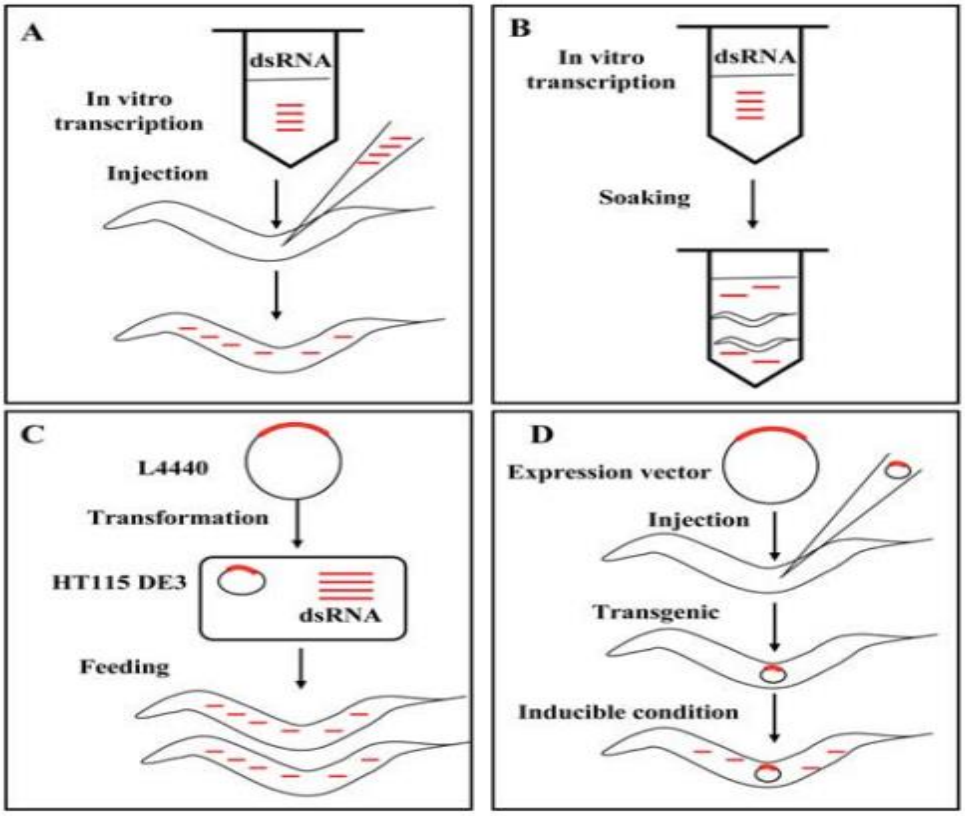

Figure 7: The major delivery methods of RNAi. (A) injection, (B) soaking, (C) feeding, and (D) transgenic (Min & Lee, 2007)

1.3.3.5 Delivery of dsRNA Through Injection

The first time the RNAi experiments were conducted, it was delivered into *D. melanogaster* hemocoel by direct injection (Kennerdell & Carthew, 2000; Dzitoyeva et al., 2001; Bettencourt et al., 2002; Quan et al., 2002). This method shows a promising approach for initiating RNAi effects since it requires only small amounts of dsRNA and it allows for the delivery of dsRNA to specific gene targets (Bettencourt et al., 2002; Amdam et al., 2003). As an advantage, it delivers the dsRNA directly to the target tissue, which provides high efficiency in inhibiting gene expression (Katoch et al., 2013) and knowing the exact amount of dsRNA inserted to the organism (Yu et al., 2013).

On the other hand, this method has a disadvantage in stimulating the immune function during the cuticular damage, which can affect and complicate the results (Han et al., 1999; Yu et al., 2013). The challenges of the injection technique are time-consuming and need more professionalism and accuracy. Therefore, another delivery method should be developed to use RNAi strategies in controlling insect pests.

1.3.3.5.1 Oral Delivery/Ingestion of dsRNA

The second delivery method is the oral method. Delivering dsRNA through feeding is the most popular oral method. It was examined firstly in nematode *C. elegan* (Timmons & Fire, 1998); after its success, it was tested in other insects. Many insect orders, such as Hemiptera (*Nilaparvata lugens*), Coleoptera (*Diabrotica virgifera virgifera*), and Lepidoptera such as *Epiphyas postvittana* and *Plutella xylostella,* proved the efficiency of gene knockdown through feeding (Mao et al., 2007; Turner et al., 2006; Baum et al., 2007; Gong et al., 2011; Chen et al., 2010).

Another way of oral delivery is droplet feeding, where the systemic RNAi exist, the dsRNA persistence across the life stage from larva to adult (Katoch et al., 2013). This method's advantages are it can minimize risk to an insect body and is easy to apply in insects that are difficult to inject. It also stimulates the natural way of feeding to the insect to make the dsRNA delivery much easier (Chen et al., 2010).

Some factors can affect RNAi's efficiency by this delivery method, such as the difficulties of determining the quantity of the dsRNA get into an insect (Surakasi et al., 2011). Another complication with this dsRNA delivery method is the requirement of a greater amount of material for delivery (Chen et al., 2010). Also, some insect species' sensitivity to RNAi molecules when they are delivered orally, was noted in *Glossina morsitans morsitans* (Walshe et al., 2009). When the dsRNA cannot inhibit

the expression of the transfer of the gene 2A192 in fat bodies. In contrast, it inhibits the expression of TsetseEP in the midgut effectively, which could refer to the shortage of transfer capacity between tissues (Walshe et al., 2009).

1.3.3.5.2 Delivery of dsRNA Through Soaking

RNAi delivered with the soaking or spraying method showed a proper response of triggering RNAi in the organism. Eaton et al. (2002) and Timmons and Fire (1998) reported that dsRNA's direct spraying has a convenient efficiency as the soaking. Tabara et al. (1998) reported the first observation of the dsRNA delivery by soaking the nematodes (*C. elegans*) in the dsRNA solution. This method is more applicable to be used in the insect cell rather than using the entire organism body. Therefore, it is used rarely.

1.3.3.5.3 Transgenic RNAi

Transgenic RNAi is one of the methods to deliver the dsRNA to the organism body *in vivo* by introducing transgenes designed for producing dsRNA of interest under the control of a heat shock promoter (Tavernarakis et al., 2000). This system was used successfully with different organism’s species including, protozoan (Bastin et al., 2001; Ngo et al., 1998), plants (Chuang & Meyerowitz, 2000; Waterhouse et al., 1998) and in insect *Drosophila* (Kennerdell & Carthew, 2000).

As each delivery method, this method has advantages and disadvantages. The advantages of this method are sustained of the transgenic lines and effects, over many generations and regulating the stage of dsRNA expression by heat shock inducible promoter. These advantages make it different from others delivery methods, and make it applicable in any situation. On the other hand, there are some limitations to this method. The efficiency of the transgene dsRNA in gene silencing is limiting to some

tissues. Moreover, the heat temperature, rising heat could affect the analysis of the phenotypes of the transgene (Min & Lee, 2007).

1.3.3.5.4 New Strategies for dsRNA Delivery

There are many more delivery methods reported and developed to increase the dsRNA's efficiency in gene silencing. Some of these methods were tested *in vitro* in certain species and show positive results while others did not. For that, further investigations need to test the promising delivery methods with less disadvantage. These methods are nanoparticle-mediated (Yu et al., 2013; Zhang et al., 2010), using biolistics (Yuen et al., 2008), electroporation, and hairpin RNA expression, using recombinant viruses (Travanty et al., 2004), and dsRNA produced in bacteria (Timmons & Fire, 1998; Wang et al., 2011).

1.3.3.6 RNAi in Insect

In the past few decades, crop losses caused by insects and the usage of pesticides costs billions of dollars every year worldwide (Katoch et al., 2013). In addition to the huge cost, it poses a potential threat of insecticide resistance development (Rodrigues & Figueira, 2016). The need to develop a new way to control the pest in an environmental approach (Katoch et al., 2013) let the researchers express the Cry toxin proteins, of *Bacillus thuringiensis* (Bt). This helps decrease the utilization of pesticides in many countries where cotton and maize are considered key crops and provide economic and environmental benefits (Amdam et al., 2003). This helps in the protection of broad categories of crops and replacing chemical insecticides (Katoch et al., 2013). Over the years, insect resistance has risen against the Bt toxins, making it necessary to develop novel and sustainable approaches to control agricultural pests (Rodrigues & Figueira, 2016). For that, research in the RNAi field

shows excellent potential because of its high specificity and might therefore serve as a new specific method to control pests in agriculture, cancers, and viral disease in medicine (Gordon & Waterhouse, 2007). The use of RNAi as a tool to examine the function of newly discovered genes has dramatically increased. With the continued sequencing of many species' genomes, higher throughput RNAi screening tools are being developed (Hannon, 2002; Kuttenkeuler & Boutros, 2004).

RNAi's mechanism showed that when the dsRNA enters the organism's body, it works in gene silencing. This helps the scientists use this method to test the efficiency in controlling the pests in different insect orders, including Coleoptera, Hymenoptera, and Lepidoptera (Camargo et al., 2016; Katoch et al., 2013; Li et al., 2011; Paim et al., 2013). The table below shows the list of insects in Coleoptera order that has been experimented with RNAi technology (Table 1).

Table 1: List of Coleoptera' insect which had successfully expression by RNAi

Order: Coleoptera			
Insect Specie / Common Name	**Target Gene**	**Application Methods**	**Reference**
Diabrotica virgifera virgifera / Western corn rootworm	Vacuolar ATPase subunit A and E; a-Tubulin	Feeding (Artificial diet)	(Baum et al., 2007)
		Transgenic plant	(Li et al.,2015)
	DvSnf7	Feeding	(Bolognesi et al., 2012)
Tribolium castaneum / Red flour beetle	eGFP	Injection	(Miller et al., 2012)
	Chitin synthase genes (TcCHS1 and TcCHS2)		(Arakane et al., 2005)
	Chitinase-like proteins (TcCHT5, TcCHT10,TcCHT7, and TcIDGF4)		(Zhu et al., 2008)

Table 1: List of Coleoptera' insect which had successfully expression by RNAi (Continued)

Order: Coleoptera			
Insect Specie / Common Name	**Target Gene**	**Application Methods**	**Reference**
Leptinotarsa decemlineata / Colorado potato beetle	Vacuolar ATPase subunit A and E	Feeding (Artificial diet)	(Baum et al., 2007)
	β-actin	Transgenic plant	(Zhang et al., 2015)
	V-ATPase E and B	Feeding	(Zhu et al., 2011)
Phyllotreta striolata / Striped flea beetle	Arginine kinase gene *AK*	Feeding	(Zhao et al., 2008)
	PsOr1	Injection	(Zhao et al., 2011)
Monochamus alternatus / Japanese pine sawyer	Laccase gene *MaLac2*	Injection	(Niu et al., 2008)
Diabrotica undecimpuctata Howardii	Vacuolar ATPase subunit A and E, a-tubulin	Feeding (Artificial diet)	(Baum et al., 2007)

1.3.3.7 RNAi Target Genes

1.3.3.7.1 Vestigial Gene (vg)

The vestigial gene (vg) is one of the members of a vestigial-like gene family (Simon et al., 2016). The vestigial gene is required for reproduction and discrimination of the adult wing and to differentiate the muscle identity of larval and adult (Pimmett et al., 2017). The loss of vestigial gene activity results in failures in wing development and ectopic gene expression and the development of ectopic wings (Williams et al., 1991; Klein & Arias, 1999).

Some studies show that vg works parallel with Scalloped protein (Sd), a member of the TEA family of transcriptional regulators, in developing wing and

haltere in insects (Halder et al., 1998). Williams et al. (1991) recognize an interaction between vg and nuclear regulatory proteins in determining which thoracic imaginal disc cells will form wings and halteres. Simmonds and his colleagues in 1998 indicated that vg and sd are working consistently to manage the expression of genes needed for wing development, which implies that vg is a tissue-specific transcriptional intermediary factor of sd (Simmonds et al., 1998). The vestigial gene is expressed in a spatially restricted pattern in the late larvae stage, identifying the subregions of these discs that will form wings and halteres (Williams et al., 1991).

In Coleopteran insects, the vestigial gene is responsible for wing development (elytra) which was proven in *D. melanogaster* and *T. castaneum* (Clark-Hachtel et al., 2013; Minakuchi et al., 2015). Elytra secure the dorsal surface from the harsh environment and protect the hindwings from damage (Andersen, 1979; Linz et al., 2016).

1.3.3.7.2 Laccase Gene (Lac)

Laccase is a member of the multicopper protein family. It is known to oxidize various aromatic and nonaromatic compounds via a radical-catalyzed reaction (Strong & Claus 2011). Laccase has been discovered in plants, animals, and microbes (Thomas et al., 1989; Claus & Filip, 1997). In insects, Dittmer and his colleagues had reported two types of laccases, Lac1, and Lac2, with different expression levels in other tissues. Lac2 is expressed primarily in the cuticle (Dittmer et al., 2004; He et al., 2007; Niu et al., 2008). Lac2 is a highly conserved multicopper oxidase, and it is expressed in the cuticle in all developmental stages in insects (Arakane et al., 2005). It plays an essential role in cuticular sclerotization and pigment synthesis (Arakane et al., 2005; He et al., 2007; Gorman et al., 2008; Niu et al., 2008). It is synthesized by the epithelial cells

and is secreted to the locations where new epidermis formation takes place to fulfill its function of cuticle melanization (Dittmer et al., 2009; Yatsu & Asano 2009; Futahashi et al., 2010). Lac2 gene expression in insects such as *Tribolium castaneum*, *Apis mellifera*, *Riptortus pedestris*, and *Drosophila melanogaster* had reduced the cuticle melanization in the exoskeleton, which leads to eventual death in some cases (Arakane et al., 2005; Niu et al., 2008; Elias-Neto et al., 2010; Futahashi et al., 2011; Riedel et al., 2011). In the case of *Monochamus alternatus*, silencing of MaLac2 gene through RNAi prove the effectiveness of this gene in obtaining successful applications in future pest control.

1.3.4 Morphological Diversity of *R. ferruginous*

Many morphological traits can be used as tools to understand the functions of coleopterans in the environment, e.g., longer body length and darker coloring, related to a higher level of plantation cover (Vandewalle et al., 2010). The color of the weevil is considered a tool to distinguish between *Rhynchophorus* species. It can be highly variable in coloration, ranging from black to reddish-brown, glossy to the matte texture (Giblin-Davis et al., 2013). The *R. ferrugineus* species is known by the orange with black markings, while a *R. vulneratus* is known by the black with a red stripe color (Hallett et al., 2004). Most of the Rhynchophorus species in the world from the species 'ferrugineus' except in the USA, California, where they have *R. vulneratus* (Rugman-Jones et al., 2013). A distinguishing key, generated by Giblin-Davis et al. (2013), helps to see differences between the adults of common *Rhynchophorus* species; it was used to eliminate any overlap due to expansion of the RPW range. In addition, body size is considered as a valuable diagnostic parameter for differences between species. Adult size is affected by egg size, larval nutrition, and developmental conditions, but

it also has a genetic basis (Beukeboom, 2018). Between *Rhynchophorus* species, 23 morphological characters were measured to distinguish between *Rhynchophorus vulneratus* and *R. ferruginous* (Sazali et al., 2018). Only three diagnostic parameters helped separate both species, which were Total Length (TL), Elytra Width (EW), and Pronotum Length (PL) (Figure 8).

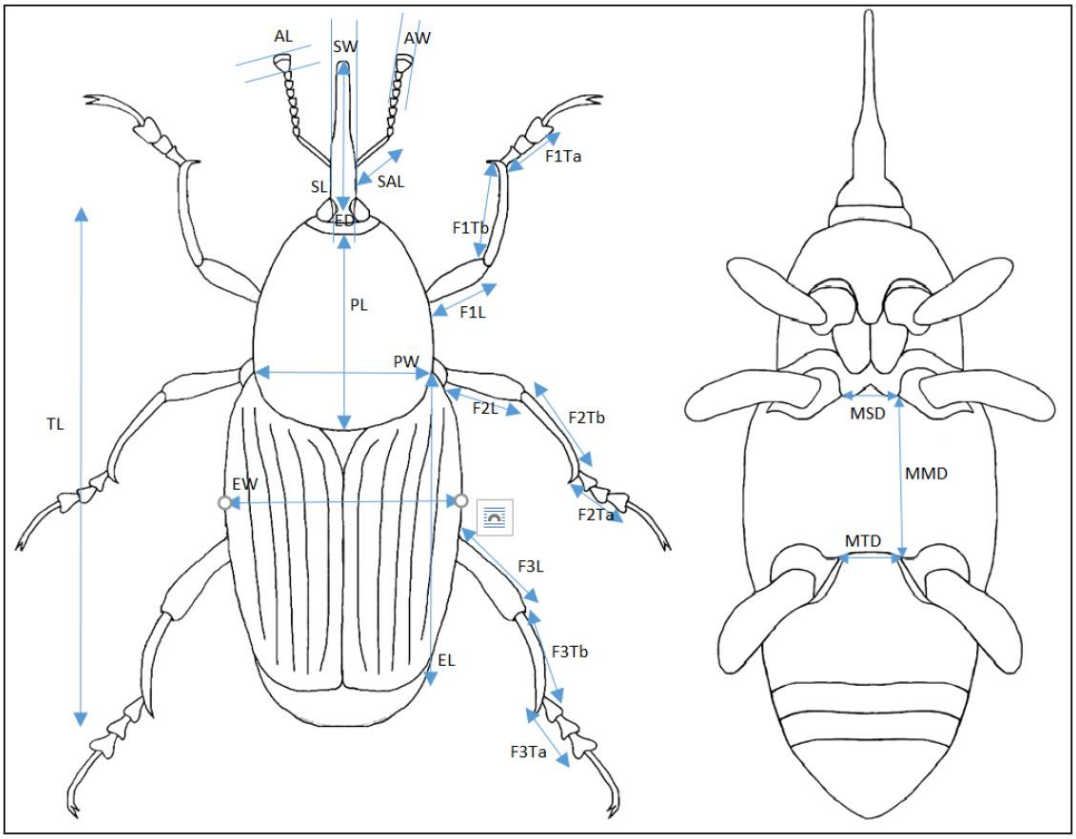

Figure 8: The morphological characters measured to compare between *Rhynchophorus* species (Sazali et al., 2018)

Many scientists also reported different numbers of male and female typologies in terms of dark prothoracic spots. In Italy, in Sicily city, Longo (2006) found eight different typologies (Figure 9), where in Malta, Mizzi et al. (2009) found ten different typologies in males and 13 in the female RPW (Figure 10). In 2018, Ul Haq et al. (2018) reported nine different typologies in males and 13 different typologies in

females (Figure 11 & 12). Sukirno et al. (2020) found 18 different color morphs of RPWs based on pronotal markings.

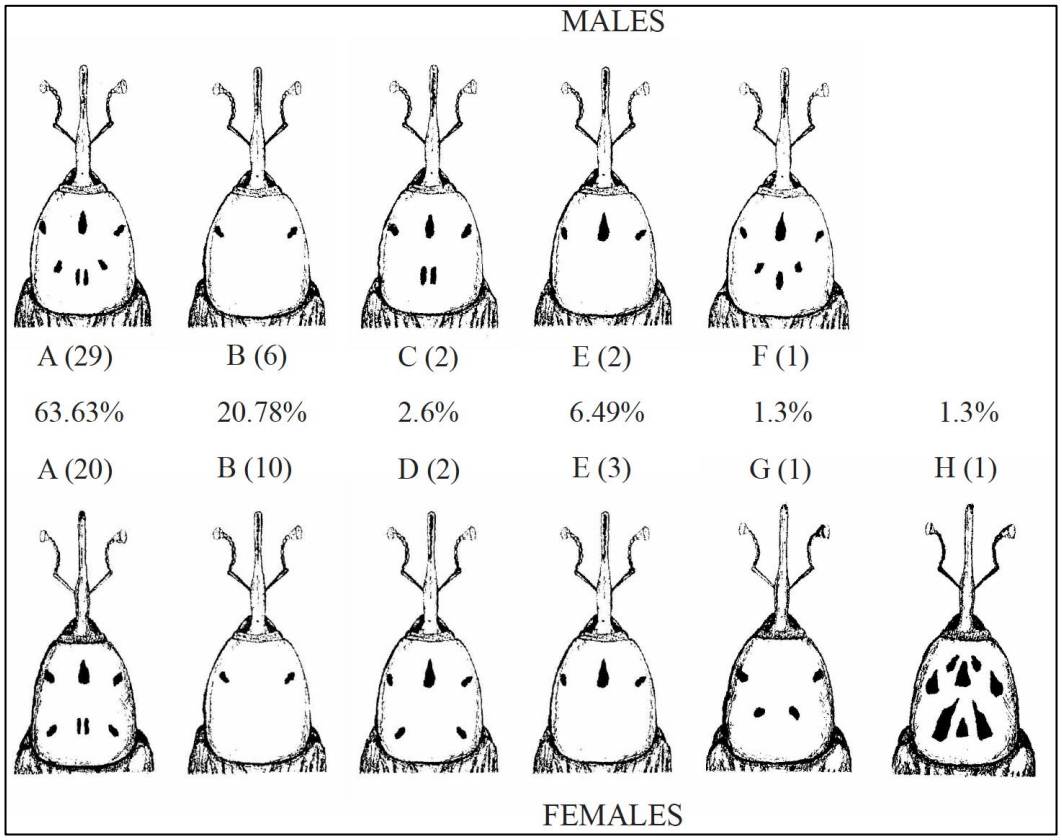

Figure 9: The different typologies of pronotal markings recorded in Sicily (Mizzi et al., 2009)

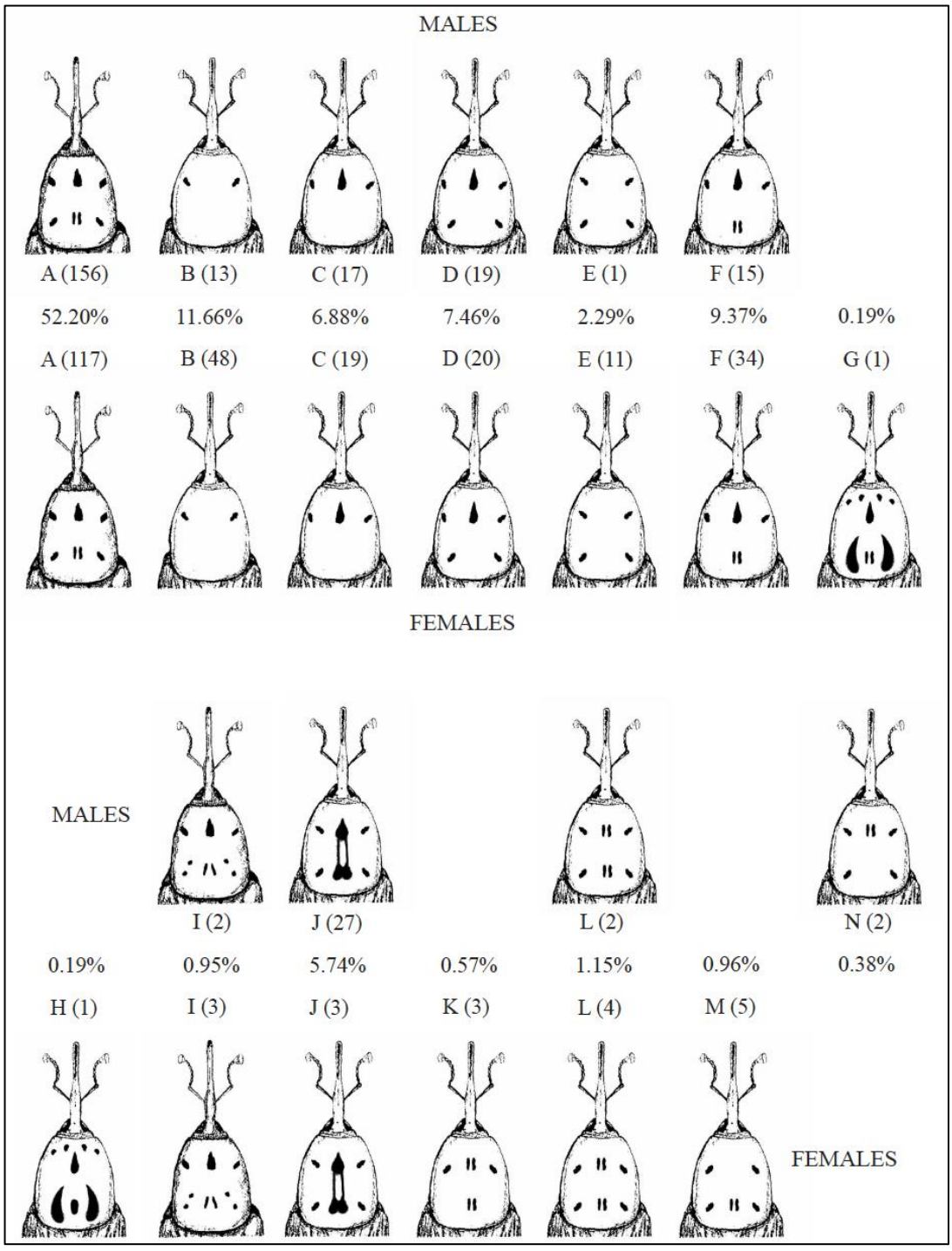

Figure 10: The different typologies of pronotal markings encountered on red palm weevils in Malta with percentage incidence (Mizzi et al., 2009)

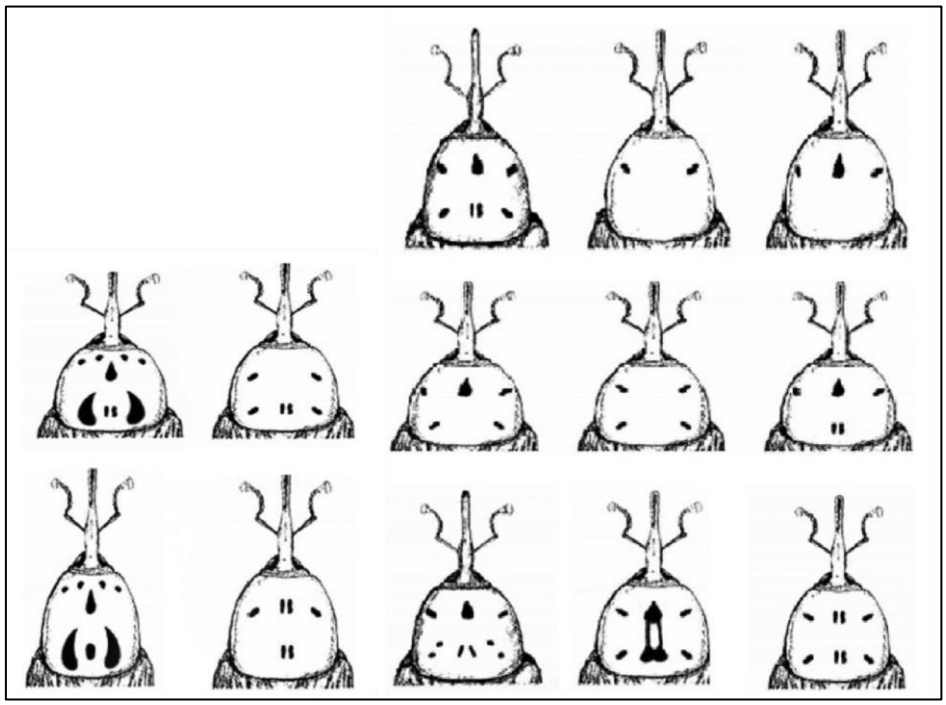

Figure 11: Typologies of prothoracic spots observed only in female in Pakistan (Ul Haq et al., 2018)

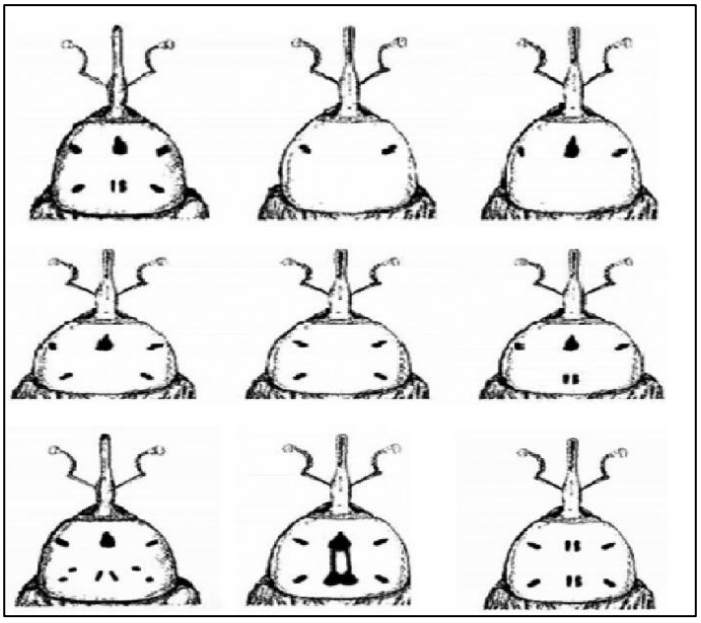

Figure 12: Typologies of prothoracic spots founded in both sexes in Pakistan (Ul Haq et al., 2018)

1.3.5 Genetic Diversity *R. ferruginous*

It is essential to know the genetic variability of the species. Environmental changes that are naturally occurring or done by human interference can affect the species (Karuppaiah & Sujayanad, 2012). Some species can adapt to the changes due to genetic diversity, and some are susceptible to death. Genetic diversity can also lead to the emergence of different individual traits, the ability to adapt to stress and disease, and environmental conditions that are not suitable for their biological nature (Muraille, 2018). This can be done in many ways, such as Cytochrome c Oxidase I (COI) DNA sequences and random amplified polymorphic DNA-PCR. The genetic analysis using a gene known as Cytochrome c Oxidase I (COI), which was established by Hebert in 2003, was used as a genetic variation tool, phylogeny, and geographical distribution in various insect species. This method aided in investigating the genetic variation of RPW from 14 invaded countries and resulted in finding eight different haplotypes.

Another way to examine the genetic diversity of the variation in red palm weevil was by using random amplified polymorphic DNA-PCR (RAPD-PCR). RAPD technique is considered a useful tool as it needs small amounts of DNA to provide rapid and specific identification of alien species (Williams et al., 1990; Hadrys et al., 1992). Many studies done by researchers using the RAPD technique were done to explore the genetic variation among diverse geographical populations of other insect species such as *Bombyx mori, Nebria gregaria, Culex quinquefasciatus, Aedes aegypti*, and *Trypanosoma cruzi* (Srivastava et al., 2005; Clarke et al., 2001; Sharma et al., 2009; Dib et al., 2009; Lala et al., 2009). Scientists studied the different morphological forms of RPW individuals in UAE, Egypt, KSA and Indonesia (Gadelhak & Enan, 2005; Salama & Saker, 2002; Al-Ayied et al., 2006). Salama and

Saker (2002) found some indications of genetic variation among three different forms of RPW. Two forms had the same RAPD amplification product, while the third group was a bit far. These differences could be due to the generation of new mutants from the weevil, or the three forms may belong to different varieties. Al-Ayied and his colleagues (2006) studied the phylogenetic relationship between the phenotypically different forms of RPW in the Al-Hasa region of the Kingdom of Saudi Arabia using the PCR-based RAPD technique. Morphologically, they found variation among the three groups of RPW, brown spotted RPW, brown non-spotted RPW, and black RPW, and genetically they were divided into two main groups with 35% genomic similarities. Moreover, by using RAPD markers, El-Mergawy et al. (2011) discovered the genetic variation and relationships within RPW population from 14 different countries. In the UAE, Gadelhak and Enan (2005) used RAPD markers to compare seven populations of RPW, and they detected 216 RAPD fragments in individuals from UAE. The populations were between 38% to 94% similarity, and thus they concluded no significant genetic variation by distance. A recent study in KSA classified RPW into three main haplotypes based on the COI gene (Sukirno et al., 2020).

Chapter 2: Methods

2.1 Red Palm Weevil Gene Silencing

2.1.1 Insect Collection

RPW larvae were collected from infested and untreated palm trees, from Al Ain City. Larvae of different instar ages were transferred to sugarcane sticks for feeding and kept under laboratory conditions.

2.1.2 RNA Extraction

Total RNA was extracted from 5 samples of the red palm weevil using an RNeasy Mini kit (Qiagen, Germany) for small tissues (< 30 mg) according to the manufacturer's instructions. RNA concentration and quality were assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA).

2.1.3 RNAi Target Genes

As it was mentioned before, two genes were selected to be targeted by RNAi in RPW. The two selected genes are affecting the insect morphologically. vg gene which is responsible for developing the wings in the insect and Lac2 which plays an important role in cuticular sclerotization and pigment synthesis. The target genes were compared with positive and negative control.

2.1.4 Template cDNA for dsRNA Synthesis

Primers were designed using Primer BLAST online tool (U.S. National Library of Medicine, 2018) (Table 2). Each primer pair amplifies DNA fragment of 400

nucleotides of the cDNA of the target gene. This region matches the mRNA region that will be targeted by the dsRNA. The 18 base pairs of the T7 promoter (TAATACGACTCACTATAG) were added to each primer to initiate the synthesis of dsRNA.

Table 2: Primers for template cDNA used in dsRNA synthesis

Primers for cDNA synthesis		
Gene Name	**Sequence**	**Length**
Vestigial gene (vg-F)	5'-**TAATACGACTCACTATAG**GGAGACGTGTCTGCAAACTGTGTCG-3' (43mer)	400 bp
Vestigial gene (vg-R)	5'-**TAATACGACTCACTATAG**GGAGACGACTCTATGGAGGGATGCTG-3' (44mer)	
laccase2 gene (lac-F)	5'-**TAATACGACACTCACTATAG**GGAGAACAGAACCTCGCCTACCTCA-3' (43mer)	400 bp
laccase2 gene (lac-R)	5'-**TAATACGACACTCACTATAG**GGAGACATTGGGTGACGAATGGCAC-3' (43mer)	
Green fluorescent protein (GFP-F)	5'-**TAATACGACTCACTATAG**GGAGACAAGGACGACGGCAACTACA-3' (43mer)	400 bp
Green fluorescent protein (GFP-R)	5'-**TAATACGACTCACTATAG**GGAGACATGCCGAGAGTGATCCCG-3' (42mer)	

cDNA was synthesized using the QIAGEN OneStep RT-PCR kit as follow, 10µl of 5x QIAGEN OneStep RT-PCR Buffer, 2 µl of dNTP Mix, 3 µl (0.6 µM) for each forward and revers primer, 2 µl of QIAGEN OneStep RT-PCR Enzyme Mix, 2 ng of RNA/reaction, and the volume was completed to reach 50 µl by adding of nuclease-free water. The PCR amplifications were conducted in 50 µl reactions and the PCR conditions were 50°C for 30 min then 94°C for 15 min, followed by 5 cycles of 92°C for 1 min, 55°C for 1 min and 72°C for 1 min, followed by 30 cycles of 92°°C for 1 min and 74°C for 1 min finishing with an extension step at 72°C for 10 min. PCR

products were purified using the OneStep PCR kit (QIAGEN). The purified PCR product was used as a template to synthesize the dsRNA.

2.1.5 dsRNA Preparation

dsRNA synthesis was carried out using MEGAscript RNAi kit (Ambion, Austin) following the manufacturer's protocol. Forward and reverse primers containing a T7 promoter site (5-'TAATACGACTCACTATAG-3') were designed to amplify a 400 bp fragment by PCR using cDNA as templates. dsRNA for Green Fluorescent Potein (dsGFP) was also produced as mentioned above and used as a negative control.

2.1.6 dsRNA Concentration

The concentration was chosen based on the RNAi studies done on the red palm weevil previously. According to Laudani et al., (2017), they used a different dose of dsRNA (1,500 ng and 5,500 ng) to investigate the gene knockdown and the morality level. In this study, 5,600 ng was the higher dose for both genes of interest, and 1,800 ng was for the vg gene as the lower dose to study the expression level and the morphological changes. The higher dose was also used for the GFP as a negative control.

2.1.7 Insect Injection

The injection was performed between the III and IV abdominal segments using the 0.5 ml micro-fine insulin syringe. Larvae were injected with 50 ul containing 1,800 or 5,600 ng of dsRNA where water colored with food dye was used a vehicle in the haemocoel around the abdomen region. Microinjection of dsGFP and nuclease-free

water was used as a negative control. After injection, larvae were kept in laboratory conditions and fed on sugarcane sticks.

2.1.8 Quantitative Real-Time PCR (RT-qPCR) for Injected Insects

Quantitative Real-Time PCR (RT-qPCR) was used to assess differential catalase gene expression in the adult stage. Total RNA of interest was extracted from the injected insect using the RNeasy Mini kit according to the manufacturer's instructions. Total RNA was quantified using Thermo Scientific NanoDrop 2000 Spectrophotometer. After that, each RNA was adjusted as 50ng in 30ul using $C_1V_1=C_2V_2$ equation. The RT-qPCR was carried out with three technical replicates. The RNA of interest and the target genes primers for RT-qPCR (Table 3) were used in Luna® Universal One-Step RT-qPCR Kit in 96 well 0.2 ml plate. A quantity of 20 µl of the reaction mixture, containing 10 µl of Luna Universal One-Step Reaction Mix (2X), 1 µl Luna WarmStart RT Enzyme Mix (20X), 0.8 µl of forward and reverse primers (10 µM), 2 µl of the RNA template, and 5.4 µl of nuclease-free water. The RT-qPCR cycling conditions were as follows: 1 cycle of 55°C for 10 min, 1 cycle of 95°C for 1min, followed by 40-45 cycles of 95°C for 10 s and 60°C for 30 s. For the melting curve stage, we followed the real-time instrument recommendations.

Table 3: Primers used in RT-qPCR

Primers for Quantitative Real-Time PCR (RT-qPCR)		
Gene Name	**Sequence**	**Length**
Vestigial gene (vg-F)	5'-TCTCGTTCTGGGACAGTCAGC-3' (20 mer)	150 bp
Vestigial gene (vg-R)	5'-TGATGGTGGTAGTCGTGGAC-3' (20 mer)	
laccase2 gene (Lac-F)	5'-CACTGGAGCACTACAGCGTT-3' (20 mer)	150 bp
laccase2 gene (Lac-R)	5'-TACTGGGACCTGGTAACATACG-3' (22 mer)	
β-actin- F	5'-AAAGGTTCCGTTGCCCTGAA-3' (20 mer)	150 bp
β-actin- R	5'-TGGCGTACAAGTCCTTCCTG-3' (20 mer)	

2.1.9 Data Analysis

A comparative Ct analysis was calculated in the QuantStudioTM 5 System instrument. The β-actin housekeeping gene was used as the endogenous control in the RT-qPCR. All results were normalized to the β-actin amplification and calculated using 2ˆΔΔCt method. Fold difference in gene expression was calculated and the percentage of downregulation (gene silencing) was assessed for each one of the tested genes. All samples were measured as duplicates.

2.2 Morphological Differences in Adult Red Palm Weevil

2.2.1 Sample Collection

Samples were collected from baited pheromone traps, and adult samples were collected weekly. Traps were integrated with a commercial lure for *R. ferrugineus*, ethyl-acetate synergist, and 200g fermenting dates in approximately 4 L of water (Abuaglala & Al-Deeb, 2012; Al-Saoud, 2018).

2.2.2 Identification of Adults Red Palm Weevil

Differentiating between males and females was made by using taxonomic work (Wattanapongsiri, 1965). Males have hairs on the end of the rostrum, whereas they are absent the females.

2.2.3 Number of Samples by the Prothoracic Spot

The difference in the numbers of the prothoracic spots in each RPW adult was studied, analyzed, and the different typologies encountered were recorded and photographed. Insects were separated into groups based on morphological differences (number of the prothoracic spots).

2.2.4 Morphometrics Measurement

According to Sazali and her colleagues, Pronotum Length (PL), Elytra Width (EW), and total length without the rostrum (L), were the most significant parameters to compare between *Rhynchophorus* species (Sazali et al., 2018). In addition to these paramertes the measurement of Elytra Length (EL), Pronotum Width (PW) were taken on the adult male and female of the red palm weevil (Figure 13). All measurements were made from the dorsal surface. Body parts were measured using the image measurement software Image J.

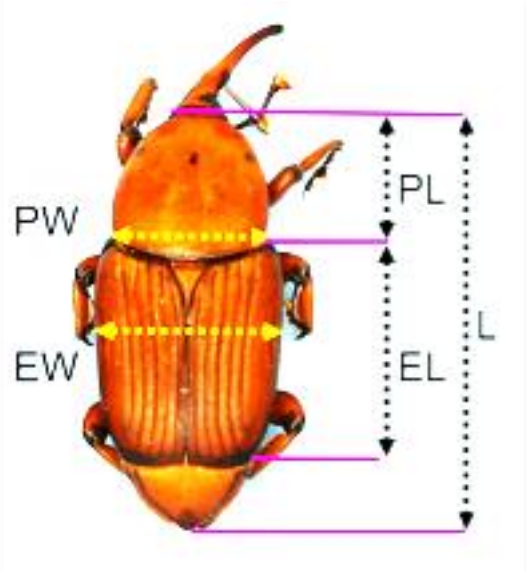

Figure 13: Body parts of the *R. ferrugineus* measured in this study

2.2.5 Rostral Setae in Male's Rostrum

Sample from the male population was taken to find the diversity in hair-like structures (rostral setae) density among the male rostrum in the UAE population. The rostral setae density in male rostrum was checked under stereoscope Leica ZOOM 2000 in magnification power 35X. The sample was divided into three categories, thick,

medium, and thin, according to the observation. A picture was taken to identify the scale.

2.2.6 Data Analysis

All collected insects were sorted based on the number of prothoracic spots (typologies of prothoracic spots). Data were presented as percentages out of the total number of insects. In terms of body part measurements, the numbers were presented in scatterplots to see data distribution within the typologies found in this study. In addition, box-and whisker plots were used to show the distribution of the body length and detect the presence of outliers. Body measurements were presented as mean ±SE (standard error) and were compared between the males and females of the different typologies. A Student *t*-test was conducted to compare the body length (L) between the 2 spots and 3 spots typologies. The percentage of each rostrum hair level of the males was calculated in the collected insects.

Chapter 3: Results

3.1 Red Palm Weevil Gene Silencing

3.1.1 Vestigial Gene Expression in the Adults of RPW

Adults emerged after injecting the larvae with dsRNA and they had a significant abnormality in wings. Morphologically, the level of the formed wing was significantly different and no hindwings appeared in the insects of the high dose, while damaged hindwings were present in the insects with the low dose (Figure 14).

Nuclease-free water	Green Fluorescent Potein	Vestigial gene low dose (1,800 ng)	Vestigial gene high dose (5,600 ng)

Figure 14: Differences in adult *R. ferrugineus* injected with vestigial gene (vg)

Relative gene expression was evaluated in the vestigial gene (vg) and control specimens of *R. ferrugineus* between the two tested doses (1,800 ng and 5,600 ng). Injecting the insects with vg dsRNA at the low dose (1,800 ng) did not affect the

transcript of vestigial protein (0.924 ± 0.228 fold, $P>0.1$) (Figure 15). On the contrary, a major reduction in expression of the vestigial gene was recorded in insects injected with the higher dose (5,600 ng) with a significant difference (0.635 ± 0.081 fold, $P<0.1$) (Figure 16). Accordingly, the vestigial gene in the injected *R. ferrugineus* specimens was 47% down-regulated relative to the control insects (injected with GFP).

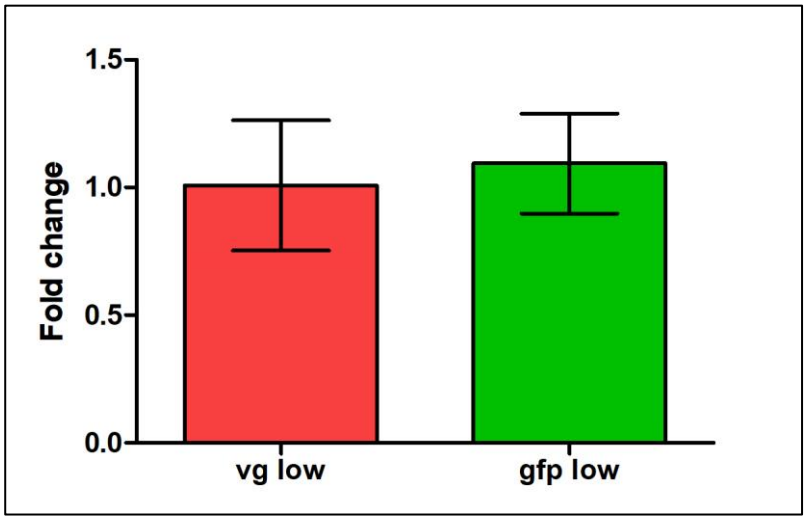

Figure 15: RT-qPCR gene expression of the vestigial (vg) gene in red palm weevils injected with (1,800 ng) dsRNA. Results are expressed as mean fold change ± SEM relative to control insects (injected with dsRNA of GFP) (n=5). Means were not significantly different. Data were analyzed by a two-tailed Student's *t*-test.

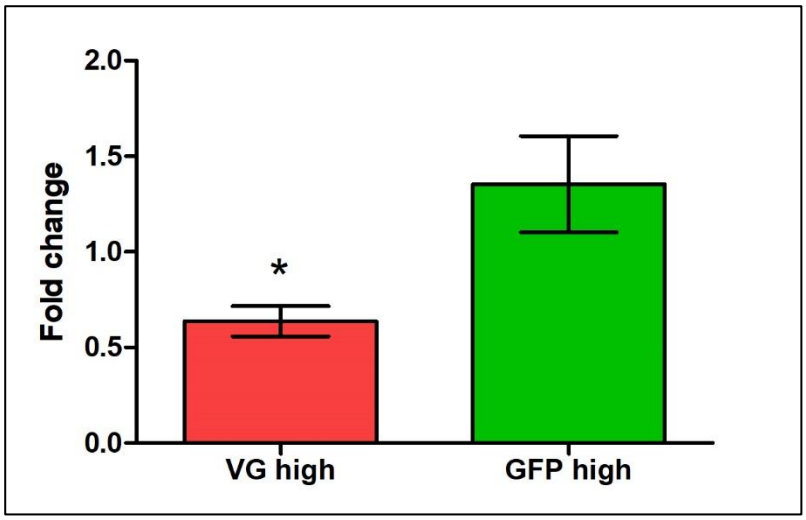

Figure 16: RT-qPCR gene expression of the vestigial (vg) gene in red palm weevils injected with (5,600 ng) dsRNA. Results are expressed as mean fold change ± SEM relative to control insects (injected with dsRNA of GFP) (n=7). * $P \leq 0.1$ indicates significant differences. Data were analyzed by a two-tailed Student's t-test.

3.1.2 Laccase Gene Expression in the Adults of RPW

Larvae that were injected with dsRNA for Laccase gene when they matured into adults significant developmental abnormalities were noticed such as soft cuticles, weak legs with difficulties in moving and standing, no stretching in the elytra wings, and damaged hindwings which are using for flight (Figure 17)

Nuclease-free water Green Fluorescent Potein Laccase gene (5,600 ng)

Figure 17: Differences in adult *R. ferrugineus* injected with Lac2 gene (Lac)

Relative gene expression was evaluated in the control and Lac2 gene (Lac) specimens of *R. ferrugineus*. The Lac gene expression appeared down-regulated with a significant difference (0.547 ± 0.091 fold) compared to the control (Figure 18). After

normalization, the Lac gene in the injected *R. ferrugineus* specimens was 57 % down-regulated relative to the control insects (injected with GFP).

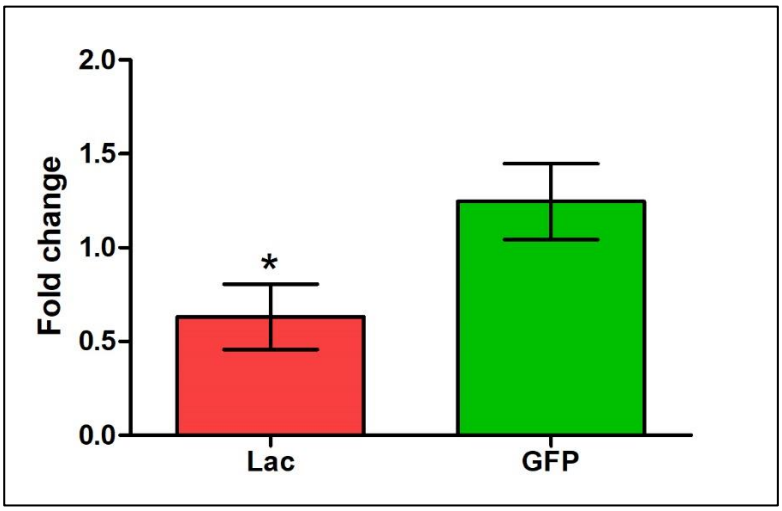

Figure 18: RT-qPCR gene expression of the laccase (lac) gene in red palm weevils injected with (5,600 ng) dsRNA. Results are expressed as mean fold change ± SEM relative to control insects (injected with dsRNA of GFP) (n=11). * $P \leq 0.1$ indicates significant differences. Data were analyzed by a two-tailed Student's t-test.

3.2 Morphological Differences in Adult Red Palm Weevil

3.2.1 Identification of Adults Red Palm Weevil

In the current study, adults of *R. ferrugineus* were collected from the aggregation pheromone trap in Al Ain city, from Feb 2018 to Feb 2019. Both alive and dead adult RPW were collected and kept in the freezer at -20°C. The total number of the collected adults was 1,269 RPW males and females and the insects were divided according to their gender.

3.2.2 Number of Samples by the Prothoracic Spot

Out of a total of 1296 adults of *R. ferrugineus* collected during this study, 602 were males while 694 were females. Seven spot typologies were recorded in this study

(Figure 19). The majority of insects belonged to the two spots typology, which was followed by the three and four spots, respectively (Figure 20). Other spot patterns existed in significantly lower prevalence. The results show that 87.0% of the females and 72.6% of the males were from the two spots typology (Table 4).

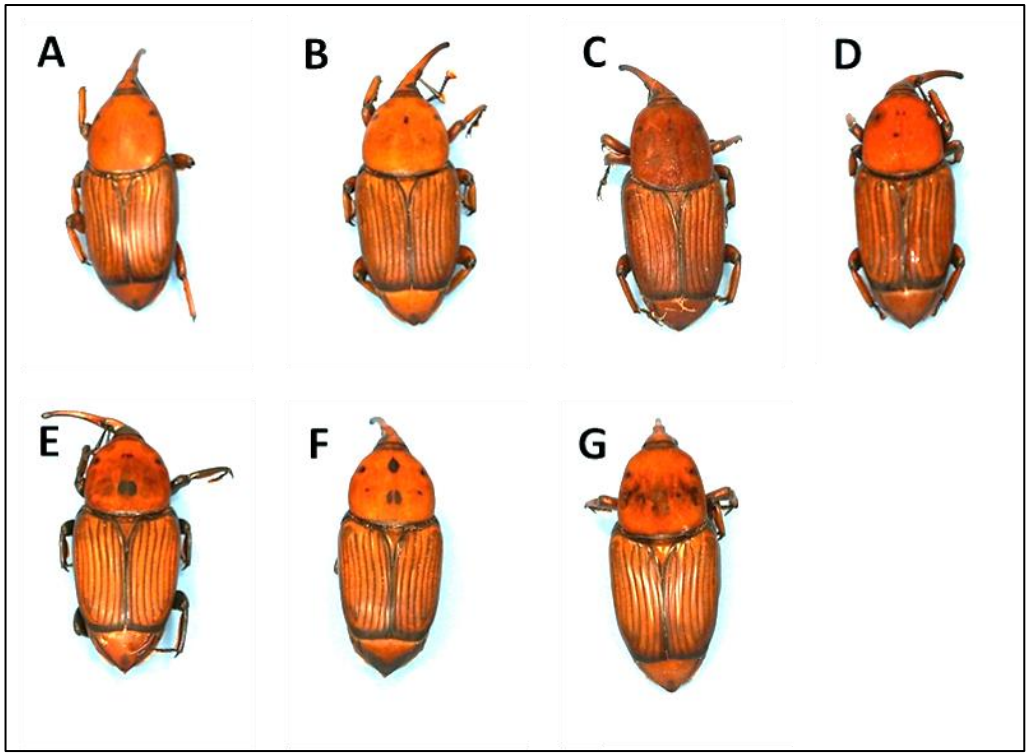

Figure 19: The seven typologies of prothoracic spots in adult *R. ferrugineus* collected in this study. (A) 2 spots, (B) 3 spots, (C) 4 spots, (D) 5 spots, € 6 spots, (F) 7 spots, (G) 8 spots.

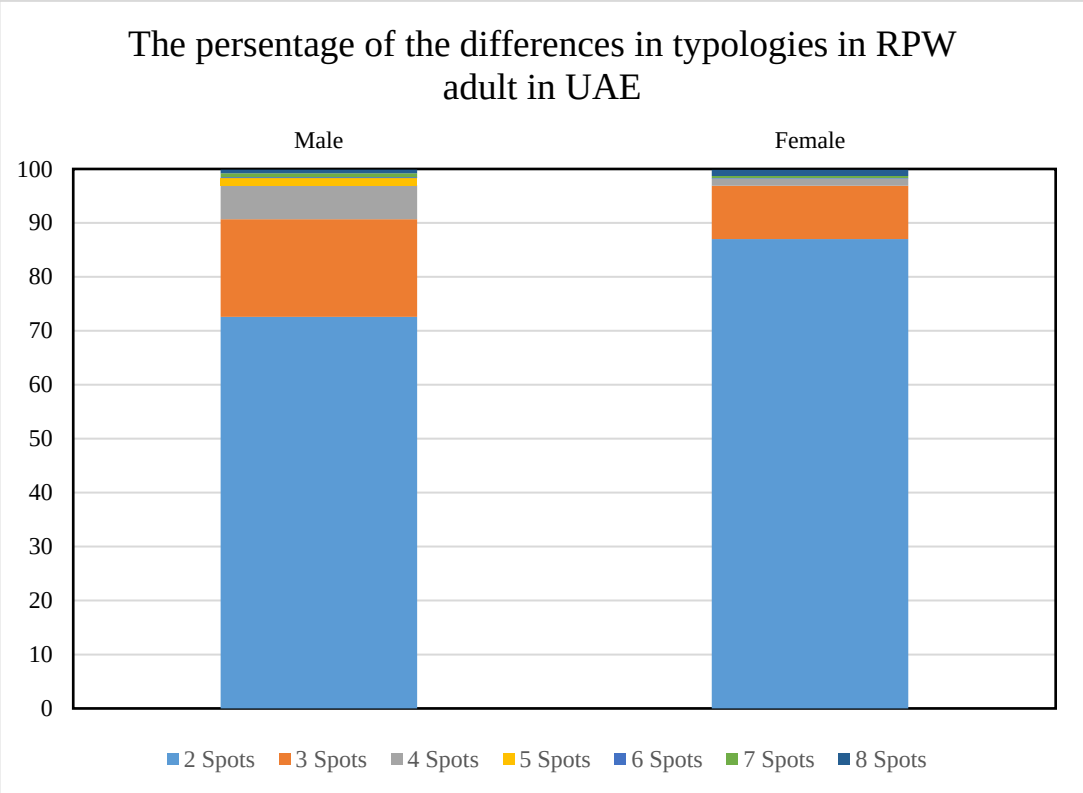

Figure 20: The percentage of the typologies of prothoracic spots in male and female adult *R. ferrugineus* in UAE(n= 602 males, n= 694 females).

Table 4: Typologies of prothoracic spots in male and female adult *R. ferrugineus*. (n= 602 males, n= 694 females)

Typology	Male %	Female %
2 Spots	72.6	87.0
3 Spots	18.1	9.9
4 Spots	6.3	1.3
5 Spots	1.3	0.0
6 Spots	0.2	0.1
7 Spots	0.7	0.4
8 Spots	0.8	1.2

3.2.3 Morphometrics Measurement

The morphometrics measurement of adult RPW males and females was taken and analyzed. All sizes were displayed in mm units. The descriptive statistics show

that insects with three and four spots are bigger than the insects with two spots in terms of the mean of body length (Table 5). In terms of body length, the insects with two spots had a wider range of body length compared to the insects with three spots and four spots for both the male and female insects (Figure 21). The results show that there were outlier points in the body length of both the males and females as shown in the box-and-whisker plots and they were mainly in the insects with three spots (Figure 22). In both males and females, insects with three spots are significantly bigger than the insects with two spots (Figure 23).

Table 5: Body measurements (mm) of *R. ferrugineus* adult males and females from different spot typologies

Gender	Spots No.	Insects No.	PL	PW	EW	EL	TL	
Female	2	96	5.25	4.992	6.023	7.568	14.15	Min
			13.13	12.38	15.52	17.83	32.73	Max
			9.21	8.837	10.66	13.21	24.58	Mean
			0.2133	0.1885	0.2213	0.2592	0.4707	SEM
Male	2	85	6.329	6.087	7.44	9.179	17.19	Min
			13.24	12.55	16.23	18.24	32.7	Max
			9.82	9.284	11.32	13.75	25.03	Mean
			0.207	0.185	0.2264	0.2548	0.4242	SEM
Female	3	37	7.536	7.15	8.744	10.68	19.84	Min
			13.03	12.25	14.3	17.64	32.35	Max
			10.85	10.18	12.16	15.18	27.69	Mean
			0.2301	0.2022	0.2381	0.2852	0.4917	SEM
Male	3	29	8.497	8.627	10.07	12.75	22.35	Min
			12.81	11.5	13.86	17.12	31.11	Max
			11.02	10.25	12.29	15.22	27.48	Mean
			0.1912	0.1469	0.165	0.2121	0.372	SEM
Female	4	4	10.48	9.58	11.26	13.48	25.1	Min
			11.26	10.18	12.98	16.08	29.28	Max
			10.77	9.852	12.05	15	27.61	Mean
			0.1766	0.1644	0.376	0.5479	0.9106	SEM
Male	4	4	5.862	5.636	6.699	8.341	15.07	Min
			12.16	11.57	13.86	17.71	30.54	Max
			10.68	9.998	11.88	14.93	27.03	Mean
			0.3278	0.3113	0.3577	0.443	0.7868	SEM

Note: Pronotum Length (PL), Pronotum Width (PW), Elythra Length (EL), Elythra Width (EW), Total Length (without rostrum) (L)

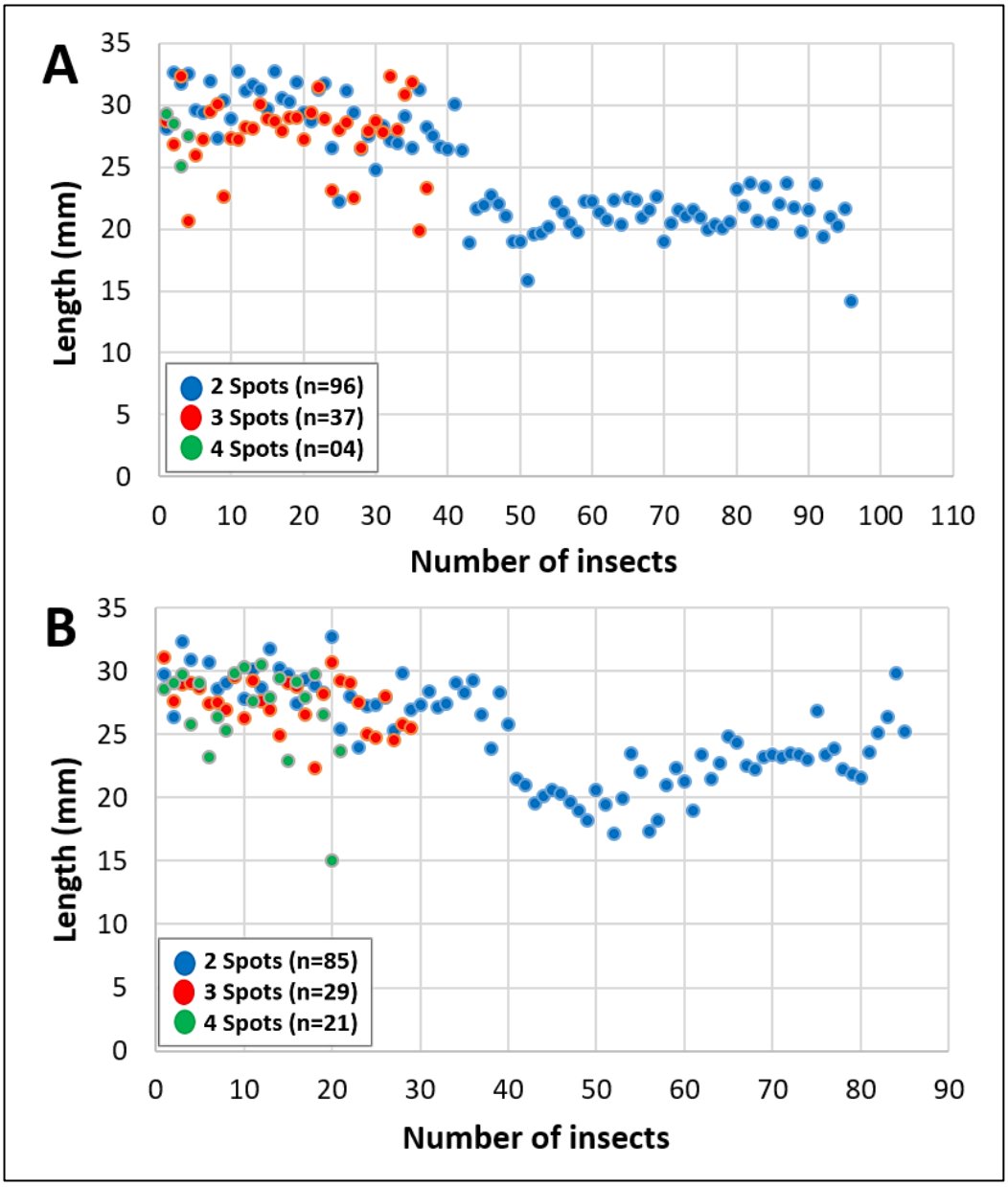

Figure 21: Scatter plot showing the distribution of body length (TL) of all females and males of *R. ferrugineus*. (A) females and (B) males.

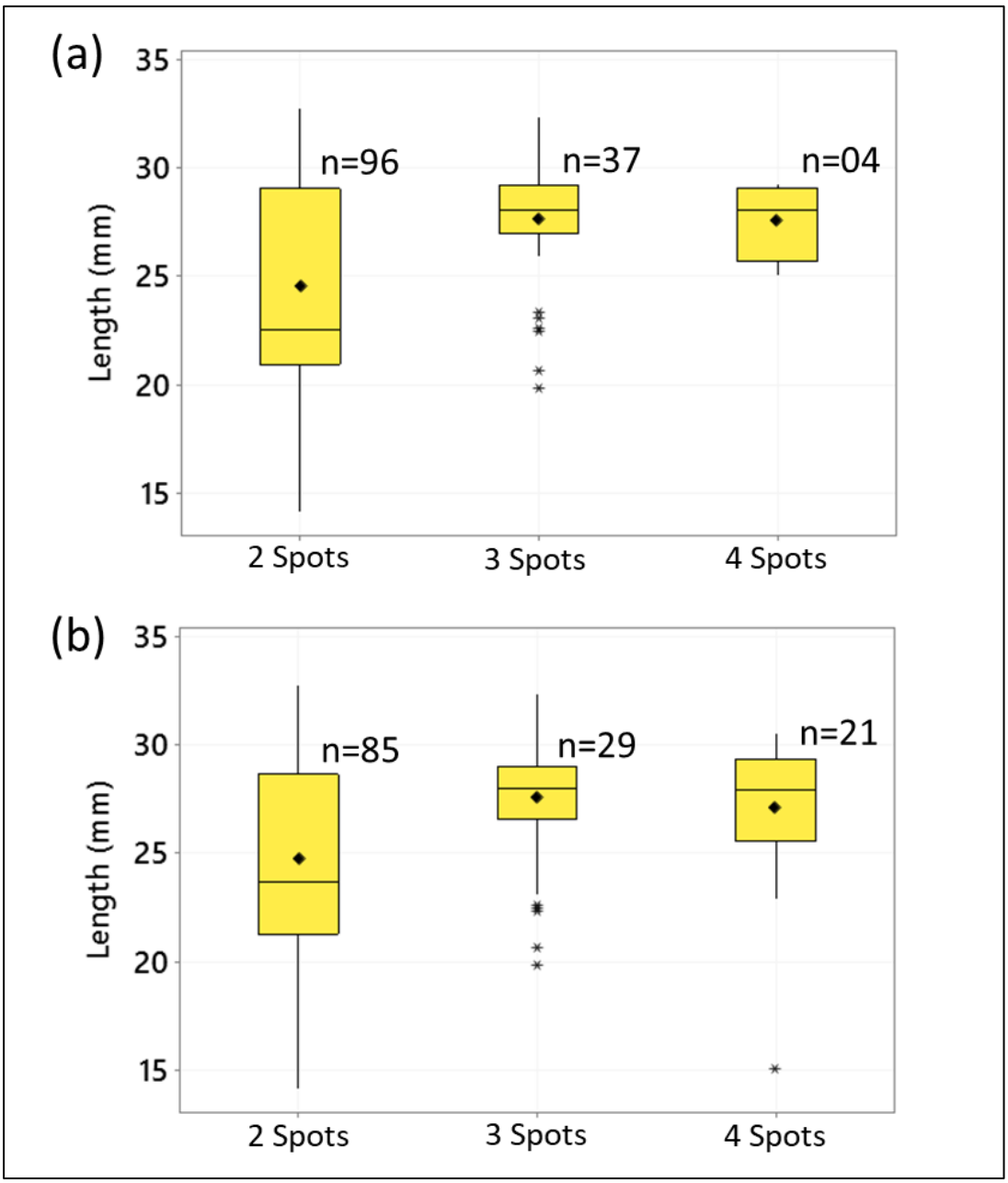

Figure 22: Box-and whisker plots showing the distribution of body length (TL) of all females and males of *R. ferrugineus*. (A) females and (B) males. *Represent the outlier values in the data

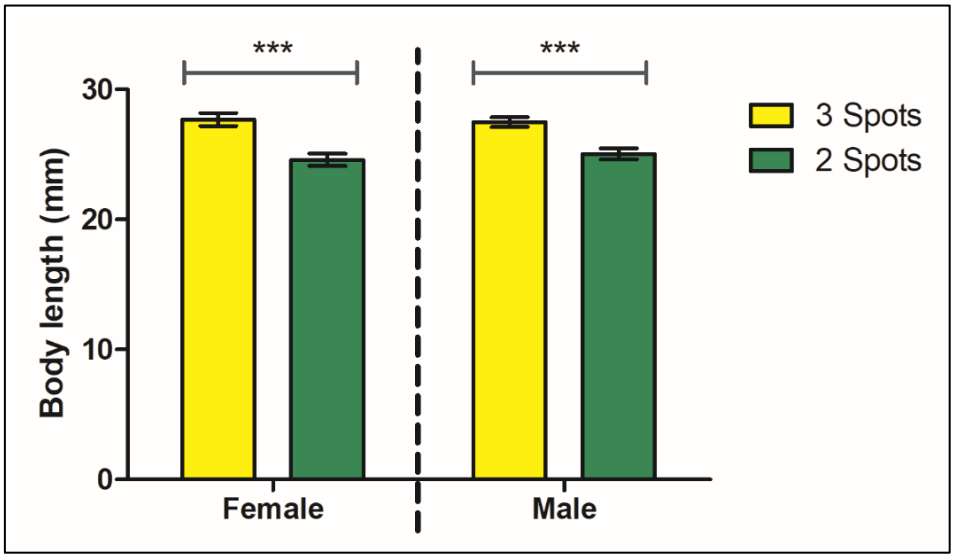

Figure 23: Comparison of the body length (L) between the 2 spots and 3 spots typologies of *R. ferrugineus* using t-test, $p<0.0001$

Comparing the results of pronotum length and width in *R. ferrugineus* adult among the UAE sample with the results of Malta and Pakistan, the UAE *R. ferrugineus* pronotum was shorter and wider than the other in both genders (Figure 24). Moreover, *R. ferrugineus* adults in the UAE population were smaller in all body measurements expect the Elytra Width (EW) in size in males and females when compared with Malaysian population (Figure 25).

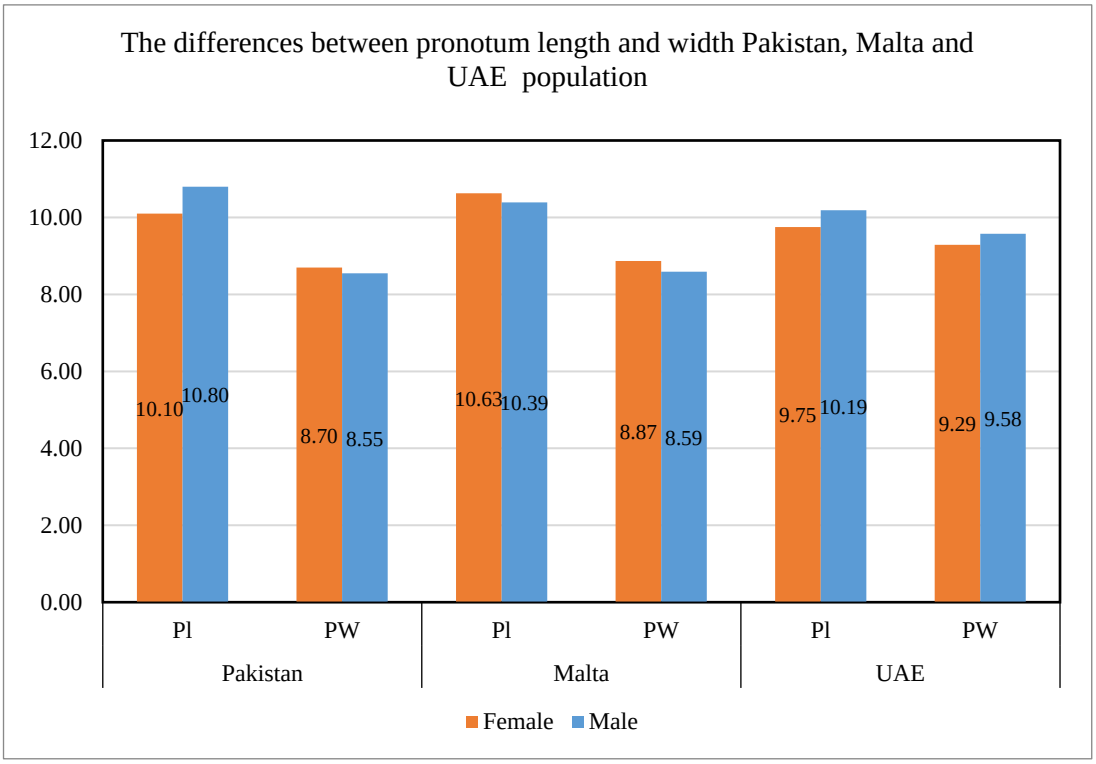

Figure 24: The different between pronotum length and width among in adult Pakistan, Malta, and UAE population

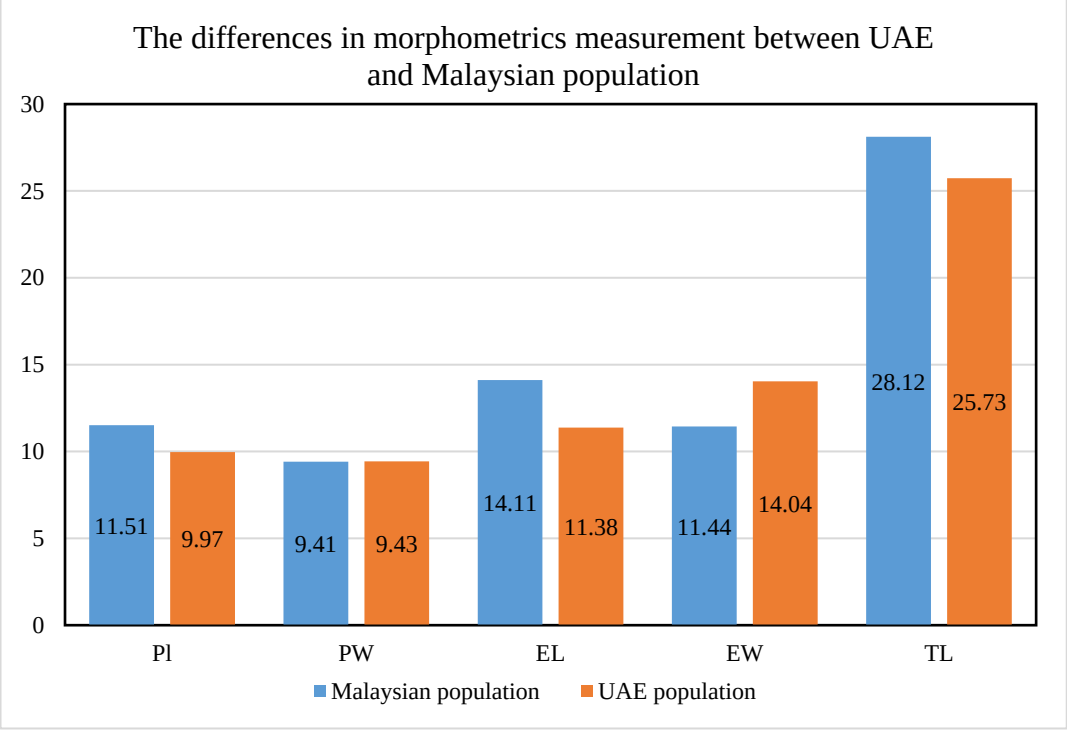

Figure 25: The differences in morphometrics' measurement between UAE and Malaysian *R. ferrugineus* population

3.2.4 Rostral Setae Density in Male's Rostrum

The results show that there are three levels of hair-like structures (rostral setae) on the rostrum of male *R. ferrugineus* and a pie chart showing the percentage of the three levels: dense (38%), medium (45%), and thin (17%) (Figure 26).

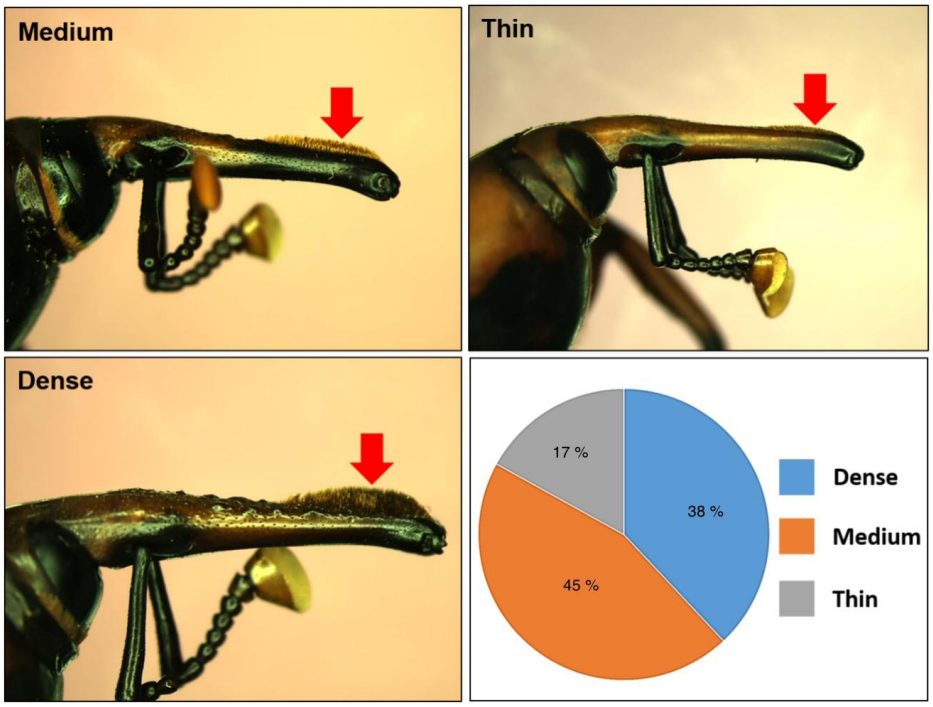

Figure 26: The three levels of setae on the rostrum of male *R. ferrugineus*. A pie chart showing the percentage of the three levels [n = 171, dense (38%), medium (45%), and thin (17%). Images on the left showing the rostrum setae levels.

Chapter 4: Discussion

4.1 Red Palm Weevil Gene Silencing

RNA interference (RNAi) has been applied as a gene silencing strategy to control pest insects by the insertion of double-stranded RNA (dsRNA). The effectiveness of RNAi differs between insect orders. It depends on the selection of the target gene, dsRNAs delivery method, dsRNAs expression, and the presence of off-target effects (Huvenne & Smagghe, 2010; Mamta & Rajam, 2017). RNAi-mediated silencing of different insect genes has been detrimental to insects' growth, development, and survival (Mamta & Rajam, 2017). This study aims to investigate the effectiveness of RNAi in *R. ferrugineus* by targeting two cuticle-related genes, vestigial and laccase genes by injecting the dsRNA. The knowledge of wing development and cuticle sclerotization in insects were limited to *Drosophila melanogaster* and *Tribolium castaneum* because of their well-established gene functional analysis tools (Arakane et al, 2004; Ohde et al., 2009). In non-model insects, gene function has been enabled through these analysis systems, thus studying gene function in new species is necessary (Ohde et al., 2009).

RNAi has been successfully achieved using different delivery methods. Furthermore, injection is one of the most popular dsRNA delivery methods and the first method used to apply RNAi (Bettencourt et al., 2002). In addition, this method has reported many advantages, as well; it has been approved that it can produce gene knockdown in *R. ferrugineus* (Laudani et al., 2017).

The vestigial gene is an essential gene responsible for wing development, so regulating this gene in the larval stage will interrupt the generation of elytra and

hindwings, which has been demonstrated in *D. melanogaster* and *T. castaneum* (Clark-Hachtel et al., 2013; Minakuchi et al., 2015). Additionally, elytra are evolutionarily modified wings, which are highly sclerotized, and act as a shield covering the dorsal surface and protecting the hindwings of the Coleoptera's insect. The presence of elytra in the insects allowed them to geographically expand, which protects them from rough environments and enhanced their ability to fly by protecting their hindwings from damage (Andersen, 1979; Linz et al., 2016). The knockdown of the vg gene was 17% and 40% downregulated in *R. ferrugineus* adults injected with 1,800 ng and 5,600 ng, respectively. This proved that regulating the vestigial gene in *R. ferrugineus* caused a significant deformity in wings along with a moving disability.

In some studies, it has been highlighted that there is no relationship between increasing the dsRNA doses and the increase of gene silencing (Meyering-Vos & Müller, 2007; Shakesby et al., 2009). In this study, using a high amount of dsRNA increased the expression of the vestigial gene slightly; this was similar to a study done by Al-Ayedh and his colleagues, who observed an increase in gene knockdown when increasing the dose of dsRNA in *R. ferrugineus* (Al-Ayedh et al., 2016). Besides this, different doses of Lac2 dsRNA were injected in *Monochamus alternates*, and the researchers found that as the dsRNA dose increased, the pigmentation and sclerotization were gradually decreased (Niu et al., 2008). In the current study, we injected the *R. ferrugineus* specimens with two different doses, 1,800 ng and 5,500 ng, to examine the different expression levels. As a result, both doses caused biological differences in the wing size and shape, however, no significant differences were noticed in the expression level of the low vg gene dose when compared to the control. This lack of statistical significance with the low dose could be related to the small sample size. Therefore, future studies should include a larger number of insects.

Laccase is one of the multicopper oxidases and has been present in the cuticles of many insect species (Yamazaki, 1972; Thomas et al., 1989). Several studies showed that the laccase-2 gene group is involved in cuticle tanning of *T. castaneum*, *N. cincticeps*, and *M. alternates*; with the highest expression levels of laccase-2 genes occuring in the epidermis (Arakane et al., 2005; Niu et al., 2008; Matsumoto & Hattori, 2018). Our data regarding the expression of *R. ferrugineus* laccase (Lac2) gene are consistent with this classification, and referring that these enzymes may have a similar function in different insects. In this study, the RNAi experiment successfully showed that the silencing of of Lac2 during cuticle sclerotization of the *R. ferrugineus* (Figure 18), produces differences in the hardness and color of the cuticle between the dsRNA-injected insects and the control insects . The knockdown of the gene was seen in a total of 57% downregulation in *R. ferrugineus*, and even it was not a 100% regulation, but it caused a morphological abnormality in adult cuticles. This result provides a clear view of how laccase (Lac2) genes play a critical role in the insect's sclerotization and pigmentation of adult cuticles, and it can be addressed as one of genes that can be used in pest control strategies.

4.2 Morphological Differences in Adult Red Palm Weevil

Studying insect diversity from the morphological aspect helps in identifying, naming, and classifying organisms; using either meristic traits or metric traits. Body features such as head and thorax are usually measured in morphometric comparisons (Tatsuta et al., 2018). Several studies have done morphometrics measurement to differentiate between *R. ferrugineus* and *R. vulneratus* (Wattanapongsiri, 1965). In addition, it has been identified that all specimens from the UAE are *R. ferrugineus* by color determination, and that they also differed significantly in pronotal shape after using the taxonomic standard measures from Wattanapongsiri's (1965) drawings. In

this research, morphological diversity was studied in *R. ferrugineus* using the differences in prothoracic spot, which were correlated with body metric traits. The results show that UAE's *R. ferrugineus* which were collected in the current study can be classified into seven typologies (Figure 19). These typologies were matched with other typologies reported in Italy, Malta, and Pakistan (Longo, 2006; Mizzi et al., 2009; Ul Haq et al., 2018). In the current study, most of the insects belonged to the two-spot typology, which was different from the results of Malta and Pakistan, where the seven-spot typology was the most common (Mizzi et al., 2009; Ul Haq et al., 2018). The presence of different typologies of prothoracic spots in many areas is indicative of the diversity of the Red Palm Weevil populations in the UAE.

In addition, the distribution of the *R. ferrugineus* in terms of body length shows that weevils in the three-spot and four-spot typologies were bigger than the weevils of the two-spot typologies, while the two spot typology weevils were varied in length with a minimum of 14.15 mm and 17.19 mm for female and male, respectively, and a maximum of 32.7 mm for both female and male. These differences are due to the sample size of the three-spots and four-spot typologies compared to the two-spot typologies sample (25, 66, 181 weevils, respectively). Adult size is affected by egg size, larval nutrition, and developmental conditions (Beukeboom, 2018). Published studies indicated that during the *R. ferrugineus* larval development, food availability affected the adult form (Wattanapongsiri, 1965).

Moreover, some studies showed how size could help the insect in a beneficial way. In general, bigger insects live longer, and a significant length in females is associated with a higher reproductive success compared to smaller individuals (Blanckenhorn, 2000; Moya-Laraño et al., 2007; Beukeboom, 2018; Tambe et al.,

2013). In addition, insect growth rates change quickly in response to environmental variation. They are closely linked to resource availability and foraging risk, thus an organism that has free access to food tends to mature faster and grows more significantly. In contrast, when food resources are limited, organisms tend to grow more slowly and reach maturity level later with a smaller size (Morey & Reznick, 2000; Day & Rowe, 2002).

The last morphometric parameter was the density of the hair-like structure, called rostral setae on the male's rostrum. Rostral setae are defined as multicellular protuberances in cuticle arthropods covering most of the body surface, and they are used for chemoreception and mechanoreception (Winterton, 2009). In insects especially, setae played a huge role in defense, and locomotion where they help some insects in walking on water, prey capture, pheromone dispersal, sexual display, preening, and camouflage. A typical seta is composed of four cells which are sensory cell, thecogen cell, trichogen cell, and tormogen cell (Winterton, 2009; Ray, 2009). Besides this, Salama and Aziz (2001) had studied the distribution of seta among the adult of *R. ferrugineus*. The abundant number of sensillae's type were identified on the antenna, mouthparts, tarsal segments and the ovipositor (Salama & Aziz, 2001).

In the current study, males of *R. ferrugineus* were divided into three levels according to the density of seta on the rostrum. About 83% of the males were between dense to medium level (Figure 28). These differences show the high ability of these males to detect the food location and availability, and helping them in finding females (Winterton, 2009). Furthermore, setae are associated with an olfactory system (sensilla setae), which is the main system that regulates sexual behavior through pheromone perception (Kruangkum et al., 2013). The *R. ferrugineus* colonizes palm trees invloves

using an odorant compound and plant volatile cues that orient the weevils towards palm trees for colonization (Antony et al., 2016; Antony et al., 2021). Additionally, these signals play a crucial role in sex pheromone perception as they use an aggregation pheromone to coordinate a mass attack (Antony et al., 2016; Kruangkum et al., 2013). As a result, the majority *R. ferrugineus* males in the UAE could be controlled by disrupting aggregation and reproduction and using early detection devices (bio-sensor) which work based on the pheromone receptor (Antony et al., 2021).

The study of adult rostral setae structure among the body surface needs to be further investigated to have clear information on setae types, their functions, or the information they provide for the guidance of behavior in addition to the work of Wattanapongsiri (1965) who has already described larval *R. ferrugineus* setae.

Chapter 5: Conclusion

In the last few decades, *R. ferrugineus* has spread widely, and as a result, has caused a severe threat to numerous palm trees worldwide. The present study combined the importance of exploring the morphological diversity of *R. ferrugineus* and observing the role of RNAi as a controlling tool in silencing two cuticle-related genes.

The morphometric *R. ferrugineus* study revealed that as with most insects, *R. ferrugineus* females have a body size larger than males. Moreover, seven prothoracic spot typologies had been found in *R. ferrugineus* of UAE. In comparison with other studies, UAE *R. ferrugineus* specimen typologies were similar to typologies reported in other countries. Although most of the insects belonged to the two-spot typology , insects with three and four spots were bigger in body size. These differences can be due to many variables such as the role of the environment, maternal age, and nutrient enrichment. In conclusion, the presence of different typologies of prothoracic spots in many areas shows the diversity of the *R. ferrugineus* populations in the UAE.

Rhynchophorus ferrugineus, like any coleopteran insect, are known for their sclerotized elytra, which protects them from rough environments and enhances their ability to fly (Andersen, 1979; Linz et al., 2016). In this study, the two principal genes of the sclerotization and forming the wings in *R. ferrugineus* were subjected to gene silencing by injecting the dsRNA. Vestigial and laccase genes have been down-regulated successfully which caused significant abnormality in wing formation, body hardnees and color. My study provides new evidence that injections of dsRNA can produce gene knockdown through RNAi in *R. ferrugineus* against the two aforementioned genes. Future studies can investigate the knockdown of these genes and their effect on the insects' physiology, the interaction between the genes, and gene

expression in response to environmental factors or hormonal signals. The successful use of gene silencing of these two genes may generate an environment-friendly pest management technique in the future.

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