El Colegio de la Frontera Sur

Detección del ADN de *Diaphorina citri* (Hemiptera: Liviidae) en el contenido intestinal de *Zelus renardii* (Hemiptera: Reduviidae)

TESIS
Presentada como requisito parcial para optar al grado de
Maestría en Ciencias en Recursos Naturales y Desarrollo Rural

por

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2016

Dedicatoria

A dios, porque no me ha dejado en los momentos que más lo he necesitado.

A mi madre por apoyarme siempre y ser mi compañera de laboratorio en las noches y madrugadas.

A mi hermano por mostrarme siempre su apoyo

Al pequeño ser vivo que se fue durante el desarrollo de esta tesis, el cual compartió conmigo 12 años de su vida y al nuevo diminuto ser que llego a reconfortarme el corazón.

Y por último, y no por eso menos importante, a MÍ el ser que más amo en este mundo, que muchas veces pensó desertar, pero supo salir adelante y aprendió que cuando las cosas se ponen más difíciles siempre hay una salida.

Agradecimientos

Agradezco a ECOSUR por haberme dado la fantástica oportunidad de estudiar la maestría, la cual ha sido una de mis mejores experiencias. También agradezco a su personal que me otorgó su amable apoyo en los momentos requeridos.

Le agradezco a CONACYT, que al otorgarme la beca por los dos años de maestría me dio la oportunidad de realizar un posgrado.

Al Dr. Juan Francisco Barrera, cuyo apoyo y confianza me dieron la oportunidad de cumplir una de mis grandes metas. Gracias Dr. Barrera por aceptarme como su estudiante.

A la Dra. Karina Guillen por su tiempo, revisiones y un espacio en el laboratorio de Biotecnología Ambiental.

Al Dr. Jaime Gómez y al Dr. Leopoldo Cruz por aceptar ser miembros de mi comité tutorial y apoyarme en el desarrollo de la tesis.

A la M. en C. Verónica García Fajardo y al M. en C. David Herrera. Maestra, gracias por su orientación, paciencia y su inmenso apoyo en el trabajo de laboratorio, aprendí mucho de usted. A David por su amabilidad, tiempo y por hacer ameno el trabajo en el laboratorio.

Al Dr. Edi Malo, por las facilidades otorgadas cuando fue Coordinador de la maestría y por estar siempre al pendiente de los estudiantes de posgrado.

Al M.E. Javier Valle Mora, por su gran apoyo en los análisis estadísticos de la tesis.

A don Quique Pascasio por ser mi compañero de campo y por estar siempre al pendiente de las necesidades del laboratorio de control biológico para que mi trabajo fuera un poco más fácil. A don Joel por facilitarme las plantas de *Murraya paniculara*. Y a la bióloga Reyna Bustamante, gracias por su amistad y por no dejar sin comer a mis chinches cuando más lo necesitaban.

Gracias a mis compañeros de generación 2014-2015, agradezco su amistad, me llevo aprendizaje de cada uno de ustedes y sin ustedes no habría pasado del primer año. Incluyo a mis amigas Amanda, Naye y May gracias por aceptarme tal como soy. Y a Hernán, Erick, Oscar, Karen, Alma y Paco, por darme momentos de recreación.

Gracias a todos

Contenido

I. Introducción

El psílido asiático, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) es el vector más importante del Huanglongbing (HLB), la enfermedad más devastadora de los cítricos en el mundo, cuyo principal agente causal en América es *Candidatus* Liberibacter asiaticus (Bové 2006; Hall et al. 2012). Este psílido es un insecto que se alimenta de la savia de plantas de la familia Rutaceae, particularmente de los géneros *Citrus* y *Murraya*; se desarrolla en los brotes y pasa por cinco instares ninfales antes de transformarse en adulto al cabo de 15 a 47 días, dependiendo de las condiciones climáticas (Morales et al. 2010).

El psílido fue detectado en México en 2002 (López-Arroyo et al. 2009) y la enfermedad fue reportada por primera vez en 2009 (Salcedo et al. 2010). México se encuentra entre los cinco primeros países productores de cítricos a nivel mundial (FAO 2012), por lo que para hacer frente a la amenaza que representa el HLB para la citricultura del país, el gobierno puso en marcha una campaña nacional de control basada en regular la distribución de material vegetal, destruir plantas enfermas y controlar al vector mediante control químico y biológico (Torres-Pacheco et al. 2013). Con respecto al control biológico, en México se emplea a *Tamarixia radiata* (Wasterson) (Hymenoptera: Eulophidae) en huertas comerciales sin uso de insecticidas, huertas abandonadas y zonas urbanas (Sánchez-González et al. 2015); este es un parasitoide de ninfas usado con éxito en las islas Guadalupe, Reunión y Puerto Rico (Étienne et al. 2001; Qureshi et al. 2009). Además, en México se han realizado diversas investigaciones para evaluar el impacto de depredadores (Reyes-Rosas et al. 2013) y patógenos (Gandarilla-Pacheco et al. 2013) contra *D. citri*.

Los depredadores generalistas han sido poco apreciados en los programas de control biológico; sin embargo, poco a poco se ha reconocido su importancia en los agroecosistemas, sobre todo en aquellos de cultivos anuales muy disturbados donde los enemigos naturales se ven forzados a colonizar o mantener sus poblaciones cuando las poblaciones de las plagas son muy bajas (Schmidt et al. 1998). Varios estudios muestran que en 75% de los casos, los depredadores generalistas redujeron significativamente la densidad de las plagas (Symondson et al. 2002). Uno de los grupos menos estudiado ha sido el de los depredadores del orden Heteroptera (Coll y Ruberson 1998); en este grupo de las llamadas "chinches verdaderas" se reconoce la importancia de la familia Reduviidae, tanto porque es la más grande en cuanto a depredadores terrestres de Heteroptera y porque incluye especies que depredan sobre muchos tipos de plagas en diferentes sistemas agrícolas (Ambrose 1999, 2000).

Entre los depredadores estudiados en México se encuentra *Zelus renardii* Kolenati (Hemiptera: Reduviidae), una chinche asesina generalista que fue reportada depredando adultos de *D. citri* en campo (Barrera et al. 2010). *Z. renardii* se encuentra distribuida en diferentes zonas climáticas de Estados Unidos, México y América Central, y es tolerante a condiciones adversas como altas temperaturas y escasez de alimento (Weirauch et al. 2012). El ciclo biológico de huevo a adulto de *Z. renardii* en condiciones de laboratorio dura de 30 a 45 días y pasa por cinco instares ninfales; las hembras de esta especie ponen masas de huevos que en promedio contienen de 19 a 35 (Barrera et al. 2010; Curkovic et al. 2004, Mbatal et al. 1987). Por lo general, *Z. renardii* embosca a las presas pequeñas y más móviles y ataca a las más grandes y menos móviles. El éxito de captura de presas es facilitada por una sustancia pegajosa que cubre las patas anteriores y

medias de ninfas y adultos de *Z. renardii* (Ables 1978; Law y Sediqi 2010). Ables (1978) observó que en plantaciones de algodón esta chinche se alimenta de diversas especies de insectos como larvas (Lepidoptera), adultos de moscas (Muscidae), adultos y larvas de catarinitas (Coccinellidae), larvas de *Chrysopa*, chicharritas (Cicadellidae), abejas (Megachilidae), parasitoides (Braconidae, Encyrtidae) y arañas. No obstante que *Z. renardii* se alimentaba con frecuencia de artrópodos benéficos, Ables (1978) también observó que una parte considerable de sus presas fueron insectos plaga. Estas relaciones intragremiales, con interacciones negativas y positivas entre *Z. renardii* y sus presas, cobran particular interés en una época donde el control biológico está interesado en estudiar a las comunidades de enemigos naturales nativos como uno de varios mecanismos reguladores naturales para el control de plagas (Ong y Vandermeer 2015; Symondson et al. 2002).

Las capacidades antes descritas motivan el interés por investigar la importancia relativa de *Z. renardii* como enemigo natural de *D. citri*. La evaluación de depredadores generalistas como *Z. renardii* no es fácil porque las interacciones con sus presas, con otros depredadores y con individuos de su propia especie son complejas (Symondson 2002). Existen varios métodos para estudiar y evaluar las interacciones presa-depredador, entre los que se pueden mencionar estudios de preferencia en laboratorio; tasas de alimentación y desempeño; observación directa de eventos de depredación o acumulación de restos de presas; análisis del intestino; y experimentos de campo (Greenstone 1999). Dado que los análisis de contenido intestinal causan mínima interrupción de las interacciones de la comunidad bajo estudio, pues solo requieren de breves interrupciones periódicas para la colecta de especímenes en campo, su uso se

ha incrementado (Harwood y Greenstone 2008). En especial, las técnicas moleculares están siendo empleadas para estudiar las interacciones presa-depredador mediante el análisis del contenido intestinal, por ser altamente específicas y sensibles (Sint et al. 2011).

El análisis molecular de contenido intestinal de las especies depende de marcadores específicos de fragmentos del ácido desoxirribonucleico (ADN), los cuales permiten detectar restos de la presa en el intestino del insecto depredador (Szendrei et al. 2009). Para este tipo de análisis los primers (oligonucleótidos) empleados por lo general son construidos en base al gen mitocondrial de Citocromo oxidasa subunidad I (COI) y subunidad II (COII) de la especie a identificar; por lo tanto, los protocolos de Reacción en Cadena de la Polimerasa (PCR por sus siglas en inglés Polymerase Chain Reaction) son modificados dependiendo de la presa y el depredador (Sint et al. 2011; von Berg et al. 2012).

Existen varios protocolos modificados para la detección de restos de presas y la vida media de estos restos en el intestino de depredadores (Chen et al. 2000, Greenstone et al. 2007, Weber y Lundgren 2009); la vida media es definida como el tiempo después del cual solamente la mitad de la comida ingerida puede ser detectada (Greenstone y Hunt 1993). Chen et al. (2000) aplicaron la técnica de PCR punto final para detectar la depredación de áfidos por *Hippodamia convergens* Guerin (Coleoptera: Coccinellidae) y *Chrysoperla plorabunda* Ficht (Neuroptera: Chrysopidae); de acuerdo con estos autores, la vida media del ADN de los áfidos en cada depredador fue 8.78 y 3.95 h respectivamente. También, empleando PCR punto final, Greenstone et al. (2007) determinaron una vida media de 7 h de *Leptinotarsa decemlineata* Say (Coleoptera:

Chrysomelidae) en el intestino de *Coleomegilla maculata* Degeer (Coleoptera: Coccinellidae) y 50.9 h en el de *Podisus maculiventris* Say (Hemiptera: Pentatomidae). Por otro lado, utilizando PCR en tiempo real (qPCR), Weber y Lundgren (2009) encontraron que la vida media de huevos de *L. decemlineata* fue de 59 min en larvas de *C. maculata*. Considerando que la capacidad de detección de los restos de la presa en el intestino del depredador disminuye a medida que éstos se digieren (Greenstone et al. 2007), se sugiere el uso de qPCR; éste es un método que no solo cuantifica la cantidad de ADN presente sino además es más sensible para detectar ADN degradado presente en heces e intestino de depredadores (Deagle et al. 2006).

El objetivo del presente estudio fue desarrollar un protocolo para estimar la vida media del ADN de *D. citri* en el intestino de *Z. renardii*. Los resultados de este trabajo darán pauta a realizar futuras investigaciones sobre la interacción de *D. citri-Z. renardii*, y aportar conocimientos sobre el comportamiento depredador de *Z. renardii* como enemigo natural de *D. citri*.

II.
Detección y vida media del ADN de *Diaphorina citri* (Hemiptera: Liviidae) en el contenido intestinal de *Zelus renardii* (Hemiptera: Reduviidae)

(Detection and half-life of *Diaphorina citri* DNA in *Zelus renardii* gut content)

Sometido a BioControl

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Abstract

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) is the most important vector of Huanglongbing, one of the most devastating diseases of citrus wide. In Mexico, the assassin bug *Zelus renardii* Kolenati (Hemiptera: Reduviidae) has been observed predating *D. citri* adults in the field. One of the techniques for studying prey-predator interactions is the molecular analysis of gut content. In this study, the half-life of *D. citri* DNA in the gut of *Z. renardii* using the real-time polymerase chain reaction technique with psyllid primers based on the Wingless gene was determined. Although the data varied greatly and some crossed reactions were obtained, it was possible to estimate the *D. citri* DNA half-life at 20.8 h in the gut of *Z. renardii*, while the time which a minimum detectable concentration of *D. citri* DNA lasted in the predator gut (0.16 ng/μL) was 42.1 h.

Keywords: Hemiptera, Liviidae, Reduviidae, pPCR, DNA detection, gut content analysis.

32 **Detection and half-life of *Diaphorina citri* DNA in *Zelus renardii* gut content**

33

34 **Introduction**

35 The Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) is the most important
36 vector of Huanglongbing (HLB), the most devastating disease of citrus wide. The causal agent of
37 HLB in America is *Candidatus* Liberibacter asiaticus (Hall et al. 2012). The psyllid is an insect
38 that feeds on the sap of plants of the Rutaceae family, particularly the genera *Citrus* and *Murraya*.
39 It develops on shoots of these plants, undergoing five nymph instars before becoming an adult after
40 15 to 47 days, depending on weather conditions (Morales et al. 2010).

41 The psyllid was detected in Mexico in 2002 (López-Arroyo et al. 2009) and the disease was first
42 reported in 2009 (Salcedo et al. 2010). Mexico is among the five most important citrus producers
43 in the world (FAO 2012), and to deal with the HLB threat to the country's citrus industry, the
44 government launched a nationwide campaign based on regulate distribution of plant material,
45 destruction of infected plants and biological and chemical control of the vector (Torres-Pacheco et
46 al. 2013). For biological control in Mexico, *Tamarixia radiata* (Wasterson) (Hymenoptera:
47 Eulophidae) is used in commercial orchards without insecticides, abandoned orchards and urban
48 areas (Sánchez-González et al. 2015). This parasitoid of *D. citri* nymphs is used successfully on
49 the islands of Guadalupe, Réunion and Puerto Rico (Qureshi et al. 2009). Moreover, in Mexico, a
50 number of studies have been conducted to evaluate the impact of predators (Reyes-Rosas et al.
51 2013) and entomopathogens (Gandarilla-Pacheco et al. 2013) against *D. citri*.

52 Generalist predators have not been highly regarded in biological control programs. However, their
53 importance in agroecosystems has been increasingly recognized, especially in highly disturbed
54 systems with annual crops where natural enemies are forced to colonize or maintain populations

55 when pest populations are very low (Schmidt et al. 1998). Several studies show that in 75% of the
56 cases, generalist predators significantly reduced pest densities (Symondson et al. 2002).
57 Heteroptera predators is one of the less studied groups (Coll and Ruberson 1998). Of this group of
58 "true bugs", the importance of Reduviidae is recognized because it is the largest Heteroptera family
59 of land predators and because it includes species that prey on many types of pests in different
60 agricultural systems (Ambrose 1999).

61 *Zelus renardii* Kolenati (Hemiptera: Reduviidae), is a generalist assassin bug that have been
62 observed predating on adult *D. citri* in the field (Barrera et al. 2010). This bug is found distributed
63 in different climate regions in the United States, Mexico and Central America, indicating that it is
64 tolerant to adverse conditions such as high temperatures and food scarcity (Weirauch et al. 2012).
65 The biological cycle of *Z. renardii* from egg to adult under laboratory conditions lasts 30 to 45
66 days and goes through five nymph instars. The females lay egg masses containing 19 to 35 (Barrera
67 et al. 2010; Curkovic et al. 2004). In general, *Z. renardii* ambushes small more mobile prey and
68 stalks larger less mobile prey. Its success in capturing prey is facilitated by a sticky substance that
69 covers the fore and middle legs of *Z. renardii* nymphs and adult (Ables 1978). Ables (1978)
70 observed that in cotton plantations, this bug feed on diverse insect species. Even though *Z. renardii*
71 often feeds on beneficial arthropods, Ables (1978) also observed that a considerable part of its prey
72 were insect pests. These intra-guild relationships, with negative and positive interactions between
73 *Z. renardii* and their preys, are of particular interest at a time when biological control is looking to
74 communities of native natural enemies as one of several natural regulating mechanisms for pest
75 control (Symondson et al. 2002).

76 Its aforementioned capacities motivate interest in investigating the relative importance of *Z.*
77 *renardii* as a natural enemy of *D. citri*. The evaluation of generalist predators like *Z. renardii* is not

78 easy because the interactions with their preys, with other predators and with individuals of its own
79 species are complex (Symondson 2002). There are several methods to study and evaluate the
80 interactions prey-predator, including laboratory studies of preference, feeding rate, and fitness of
81 predators; direct observation of predation events or accumulation of prey carcasses; gut analysis;
82 and field experiments (Greenstone 1999). Analysis of gut content causes minimal interruption of
83 community interactions since it requires only brief periodic interruptions for specimen collection
84 in the field (Harwood and Greenstone 2008). For this reason, its use has increased, especially
85 molecular techniques, which are being used to study prey-predator interactions by analysis of gut
86 content and which are highly specific and sensitive (Sint et al. 2011).

87 Molecular analysis of gut content of the species depends on specific markers of deoxyribonucleic
88 acid (DNA) fragments, which enable detection of prey remains in an insect predator's gut (Szendrei
89 et al. 2009). For this type of analysis, the primers (oligonucelotides) used, in general, are
90 constructed on the basis of the mitochondrial gene of Cytochrome oxidase subunit I (COI) and
91 subunit II (COII) of the species to be identified. Therefore, polymerase chain reaction (PRC)
92 protocols are modified in accord with the prey and the predator (Sint et al. 2011).

93 Protocols modified for detecting prey remains in predator have been developed previously (Chen
94 et al. 2000, Greenstone et al. 2007, Weber and Lundgren 2009). For instance, Chen et al. (2000)
95 applied the end-point PCR technique to detect predation of aphids by *Hippodamia convergens*
96 Guerin (Coleoptera: Coccinellidae) and *Chrysoperla plorabunda* Ficht (Neuroptera: Chrysopidae).
97 According to these authors, DNA half-life of aphids in the predators was 8.78 and 3.95 h,
98 respectively. Half-life is defined as the time after which only half of the ingested food can be
99 detected (Greenstone and Hunt 1993). Moreover, using end-point PCR, Greenstone et al. (2007)
100 determined a half-life of 7 h for *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) in

101 the gut of *Coleomegilla maculata* Degeer (Coleoptera: Coccinellidae) and 50.9 h in that of *Podisus*
102 *maculiventris* Say (Hemiptera: Pentatomidae). Furthermore, using real-time PCR (qPCR), Weber
103 and Lundren (2009) found that the half-life of *L. decemlineata* eggs was 59 min in larvae of *C.*
104 *maculata*. Because the ability to detect remains of prey in predator gut decreases with digestion
105 (Greenstone et al. 2007), the use of qPCR is suggested. This is a method that not only quantifies
106 the amount of DNA present, but it is also more sensitive for detecting degraded DNA present in
107 feces and gut of predators (Deagel et al. 2006).

108 The objective of this study was to develop a protocol to estimate the half-life of Asian citrus psyllid
109 *D. citri* DNA in the gut of assassin bug *Z. renardii*. The results of this study will provide guidelines
110 for future studies on *D. citri*-*Z. renardii* interactions, and contribute knowledge on the predating
111 behavior of *Z. renardii* as a natural enemy of *D. citri*.

112

Materials and Methods

Insects

Z. renardii nymphs and adults were collected from *Murraya paniculata* (L.) Jack (Rutaceae) plants in the city of Tapachula, Chiapas, Mexico. The insects were taken to the Biological Control Laboratory at El Colegio de la Frontera Sur (ECOSUR) for rearing and reproduction. Offspring were maintained at 27 ± 1°C, 80% R.H. and a photoperiod of 12:12 h light: dark. *Z. renardii* nymphs fed on adult *Drosophila* spp. reared in the laboratory and *Z. renardii* adults fed on *Anastrepha ludens* (Loew) adults from the rearing laboratory of the fruit fly program located in Metapa, Chiapas, Mexico.

Estimation of *D. citri* DNA half-life in the *Z. renardii* gut

Fifty *Z. renardii* adults were randomly selected and placed in 50 mL conical tubes (Falcon™) individually to prevent cannibalism. The tubes contained a pleated paper rectangle 9 x 3 cm as support for the insect. Each one was fed once with 20 *D. citri* adults during a period of 8 h. After this time, all of the prey, live and dead, were withdrawn. After 0, 8, 12, 24 and 48 h, the predator bugs were killed placing them at a temperature of -20 ºC to stop digestion. The insects were then stored in 70 % alcohol at -20 ºC in 1.5 mL tubes (Axygen Scientific Inc, Union City, California). DNA was extracted from the gut of these individuals to estimate its half-life.

Specificity and detection sensitivity tests

This experiment was conducted to determine the effect of the type of prey and post-feeding time on detection of *D. citri* DNA in the gut of *Z. renardii*. As in the previous case, *Z. renardii* adults were placed individually in conical 50 mL tubes containing a pleated paper rectangle 9 x 3 cm. Before the experiments, the insects were kept for 48 h without food. The experiment consisted of

feeding the bugs only once with combinations of two types of prey: adult *D. citri* (DC) and adult *Drosophila* spp. (DS). Six treatments (T) were used: T1, 30 DC+10 DS; T2, 20 DC+20 DS; T3, 10 DC+30 DS; T4, 40 DC+0 DS; T5, 0 DC+40 DS; and T6, 0 DC+0 DS. For each treatment, 50 *Z. renardii* adults selected randomly were used. Each individual was considered one replication. After 0, 8, 12, 24 and 48 h post-feeding, 10 predators per treatment and per time were killed and stored as in the previous experiment. However, in this case the live or dead preys were kept in contact with the predator bugs during the period of observation. The experiment used a total of 300 *Z. renardii* adults.

Experiment with cages

This experiment aimed to determine the effect of the type of prey and post-feeding time on *D. citri* DNA detection in the gut of *Z. renardii*. However, unlike the previous case, the experiment was conducted with 60 x 60 x 60 cm cages (BugDorm-2120 Insect Tent BD2120, MegaView Science Co. Ltd, Taiwan) and every day the dead preys were replaced by live preys. In each of the four cages, a 2 years old *M. paniculata* plant of 60-70 cm height was introduced and later four adult *Z. renardii* (2 females and 2 males). The treatments (T) consisted of releasing 160 adult *D. citri* (DC) and 16 adult *A. ludens* (AL) per cage per day for four consecutive days with the following modalities: T1 (cage 1), 160 DC per day; T2 (cage 2), 160 DC days 1-3 and 16 AL on day 4; T3 (cage 3), 160 DC on days 1-2 and 16 AL on days 3-4; and T4 (cage 4), 160 DC on day 1 and 16 AL on days 2-4. At the end of the experiment, only in cage 1 was *D. citri* present constantly. Every 24 h after initiating the experiment (24, 48, 72 and 96 h), four *Z. renardii* adults were removed at random, one per cage, and killed and stored as mentioned above until DNA extraction from the *Z. renardii* gut.

158 Primers design and validation

159 Primers for *Z. renardii* were designed (Primer_3_Zelus_Fcoi= GACTGCCCATGCATTCATTA
160 and Primer_3_Zelus_Rcoi= GTACCAGCTCCTCCTTCAGC), which aligned in a region of the
161 gene for COI with the Genbank sequence number JQ888723.1, using the software MEGA6
162 (Tamura et al. 2013) and Primer3Plus (Untergasser et al. 2007). For *D. citri*, primers of
163 glycoprotein genes were used, Wg (WGf=GCTCTCAAAGATCGGTTTGACGG and WGr=
164 GCTGCCACGAACGTTACCTTC), reported in the National Protocol of *Candidatus* Liberibacter
165 spp., with the qPCR technique (DGSV 2010).

166 Following the modified protocol of the phenol-chloroform method (Sambrook and Russell 2001),
167 *Z. renardii* DNA was extracted from one individual from which wings and feet were removed,
168 while *D. citri* DNA was extracted from 20 intact individuals. To visualize the DNA, 1 % agarose
169 gels were used; 5 µL of each sample were used, stained with SYBR Green 1000X (Invitrogen,
170 Carlsbad, California) and separated for 30 min at 90 V.

171 An end-point PCR was performed in a thermocycler (Select. BioProducts, USA) to determine the
172 optimum aligning temperature of each pair of primers. Each 20 µL PCR mix contained 1 µL DNA;
173 the reaction was composed of 1x PCR buffer (10x Taq Buffer + KCl-$MgCl_2$, Thermo Scientific,
174 Waltham, Massachusetts), 0.2 pM of each primer, 0.2 mM dNTPs (Mix 10mM Vivantis, Selangor
175 Darul Ehsan, Malaysia), 1.5 mM $MgCl_2$ (50 mM, Invitrogen, Carlsbad, California) and 1 U/µL
176 *Taq* polymerase (Invitrogen, Carlsbad, California).

177 After establish the optimum alignment temperatures, crossed tests were conducted between
178 primers. *Z. renardii* primers were used with DNA from *D. citri* as a template and vice versa. As in
179 the previous case, each 20 µL PCR mix contained 1 µL DNA and the reaction was composed of

1x PCR buffer with the characteristics mentioned above. All the samples were amplified, commencing with an initial denaturalization step of 96 ºC for 5 min, followed by 35 cycles of denaturalization at 96 ºC for 30 s, alignment at 54.4 ºC for *Z. renardii* and 58.3 ºC for *D. citri* for 45 s and extension at 74 ºC for 1 min, and one final extension at 74 ºC for 5 min in all reactions. The amplicon (2000 pb from *Z. renardii* and 200 pb from *D. citri*) was purified using the Wizard® SV Gel kit and the Clean-Up System (Promega, Madison, WI), and ligation and transformation were carried out in *Escherichia coli* DH5α cell with the plasmid pJET 1.2/blunt Cloning Vector (ThermoFisher, Waltham, Massachusetts). The plasmid was purified with the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and insert was sequenced by Sanger capillary sequencing method (Macrogen Inc., Korea). The sequences were analyzed and compared with the database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and Barcode of Life Data Systems database (http://www.boldsystems.org/).

qPCR screening

The samples of *Z. renardii* specimens were removed from the alcohol and washed twice with sterile 20 mM EDTA, pH 8. They were then placed in disposable 100 x 15 mm plastic Petri dishes with sterile 0.1 M phosphate buffer, pH 7. To extract the gut, the *Z. renardii* abdomen was cut open with manicure scissors. To facilitate the operation, egg masses from females and Malpighi tubes from the males were removed first. The gut was extracted intact and, with a scalpel, the midgut was cut and stored individually in 1.5 mL tubes (Axygen Scientific Inc., Union City, California, USA) with sterile 0.1 m phosphate buffer, pH 7. Immediately afterward, the DNA was extracted.

Because of the small size of the midgut of *Z. renardii*, DNA extraction was performed using the modified ZR Tissue & Insect DNA Micro Prep ^{TM} protocol of Zymo. To this end, the midgut was removed from the phosphate buffer and 50% of the quantity of reagents provided by the supplier

was added. Finally, the column was transferred to a clean 1.5 mL micro-centrifuge tube, and 20 µL injectable water was added directly to the column matrix and left in repose 10 min. It was then centrifuged at 10,000 x g for 1 min to elute the DNA. Three µL of this was used in electrophoresis with 1 % agarose gel stained with SYBR Green (1,000X Invitrogen) to visualize the quality of the extracted DNA, which was stored at -20 ºC for later analysis. DNA quality of each extraction was observed using the 260/280 nm absorbance obtained in the UV-1700 Pharmaspec UV-visible Spectrophotometer (Shimadzu, Kyoto, Japan). Excluding the *Z. renardii* individuals that died during the experiment, DNA was extracted from 356 individuals (97.47 %).

A calibration curve in qPCR was constructed with different concentrations of DNA from *D. citri*; 100 ng was the maximum concentration of the curve and 0.8 ng was the minimum concentration. Each DNA sample from *Z. renardii* gut was interpolated on the calibration curve to obtain the concentration of *D. citri* DNA present in the gut. The lowest detectable concentration of *D. citri* DNA in the *Z. renardii* gut (C_{md}), obtained through series of dilutions, was 0.16 ng/µL.

The reaction (12 µL of the total volume) was composed of Master Mix at 1X (SYBR® Select Master Mix 1 x 5 mL Invitrogen), 0.125 pM of each *D. citri* primer (WGf and WGr) and the DNA of each sample. To carry out the qPCR, a range of 100 to 400 ng total DNA was used. The extractions were amplified in the Rotor-Gene 6000 (Corbet Research, San Francisco), under the following conditions: 95 ºC for 5 min, followed by 40 cycles at 94 ºC for 15 s and 60.5 ºC for 60 s. The chromophore used was SYBR Green.

Data analysis

The relationship between the *D. citri* DNA concentration in the *Z. renardii* gut (C), relative to time (t) was established by means of the non-linear regression model $C = a + b\, e^{ct}$, where a, b and c are

225 coefficients estimated by interactions, $e = 2.71828$ and $t = 0$, 8, 12, 24 and 48 h. With this equation,
226 we solved for t to estimate the half-life of *D. citri* DNA in the *Z. renardii* gut (t_{50}) with the equation
227 $t_{50} = (\ln [(C_{50} - a)/ b]) / c$. Moreover, the maximum time (t_{max}) in which a minimum concentration
228 of *D. citri* DNA can be detected in the *Z. renardii* gut ($C_{md} = 0.16$ ng/µL) was estimated with the
229 equation $t_{max} = (\ln [(C_{md} - a)/ b]) / c$.

230 Because of the variability in the values of the *D. citri* DNA concentrations in the gut of adult *Z.*
231 *renardii* obtained by qPCR in the specificity and detection sensitivity tests, ranges of data were
232 transformed to obtain robust comparisons of the treatments and observation time (Conover and
233 Iman 1981). The data corresponding to time 0 h of each treatment were discarded because they
234 varied highly. A bifactorial (feeding treatment x post-feeding time) analysis of variance (ANOVA)
235 was performed and treatments and times were then compared by orthogonal contrasts with the
236 Bonferroni correction.

237 Data from the cage experiment were subjected to an ANOVA and a Tukey test (5%) for comparison
238 of treatment means.

239 **Results**

240 Primers design and validation

241 By comparing the *Z. renardii* and *D. citri* sequences with the NCBI and Boldsystems databases,
242 an identity of 100% was obtained for the two species, and when the crossed tests were conducted
243 with end-point PCR, there was no amplification of the *D. citri* primers with the DNA from *Z.*
244 *renardii*, nor of the *Z. renardii* primers with the DNA of *D. citri*, confirming the specificity of the
245 primers used.

246

D. citri DNA half-life in the *Z. renardii* gut

The equation parameters that best described the relationship between concentration of *D. citri* DNA present in the *Z. renardii* gut and the time lapsed after the predator bugs fed was $C = 1.02871 + (-0.5)\ e^{0.01312\ t}$. This relationship, however, was not significant ($P>0.05$, $R^2= 0.20$; Fig. 1). Nevertheless, when *t* of this equation was solved for, it was possible to estimate *D. citri* DNA half-life in the *Z. renardii* gut at 20.8 h for $C_{50} = 0.372$ ng/µL. The maximum time a minimum detectable concentration of *D. citri* DNA is present in the *Z. renardii* gut, according to the test of serial dilutions (0.16 ng/µL), was estimated at 42.1 h.

Specificity and detection sensitivity tests

According to the ANOVA, there were significant differences among treatments or types and quantities of prey offered to *Z. renardii* (df=5, F=42.08, P<0.001), among the periods of time lapsed or sample collection after feeding (df=3, F =2.97, P=0.0329) and their interaction (df=15, F=5.75, P<001).

All of the treatments were *D. citri* positive in the qPCR test, even those whose predators were not fed this prey, as in T5 (0 *D. citri*, 40 *Drosophila* spp.) and (0 *D. citri*, 0 *Drosophila* spp.) (Table 1). In general, there was high variability in the treatments, making it difficult to detect any particular tendency, although T5 and T6 had the highest responses, and T3 (10 *D. citri*, 30 *Drosophila* spp.) and T4 (40 *D. citri*, 0 *Drosophila* spp.) had the lowest. T4, the treatment in which the predators were given only *D. citri* was statistically different (P<0.05) at all sampling times from T5, the treatment in which the predator received only *Drosophila* spp., and statistically different (P<0.05) from T6 only at 12 and 48 h. As expected, T4 was statistically similar (P>0.05) to the other treatments in which *D. citri* was supplied (T1-T3), although this was observed only at 8 and 12 h.

269 In terms of sampling times, the exception was T4 (40 *D. citri*, 0 *Drosophila* spp.), in which, as of
270 12 h, a significant decreasing trend was observed in the concentration of *D. citri* DNA in the gut
271 of *Z. renardii*. In all of the other treatments, there were no significant changes over time (Table 1).

272 Experiment with cages

273 There were no significant differences in *D. citri* DNA concentration in the of *Z. renardii* gut among
274 sampling times: 24, 48, 72 and 96 h (F=1.5538, df=3, P=0.2744). There were differences, however,
275 among cages (F=254.1, df=3, P<0.001). According to the 5% Tukey test, the concentration of *D.*
276 *citri* DNA in the gut of *Z. renardii* individuals in cage 3 (T3) that received 320 *D. citri* and 32 *A.*
277 *ludens* for four days was higher than that found in individuals in the other three cages. The *D. citri*
278 DNA concentration in the gut of *Z. renardii* individuals in cage 1 (T1) fed only *D. citri* (640 DC
279 for four days) was statistically equal to that of individuals in cages 2 and 4, while between these
280 two cages there were no differences (Table 2).

281 **Discussion**

282 Prey-predator interactions are complex primary processes that control the changes in animal
283 populations and, therefore, understanding them is fundamental in many ecological and biological
284 control studies. In the case of generalist predators, identification and quantification of these
285 interactions is more complex because these predators feed on a broad spectrum of prey (Symondson
286 2002). The study of these interactions can be facilitated by specific sensitive molecular techniques
287 (Sint et al. 2011), such as qPCR, and specific primers of the target prey to be identified. For
288 example, COI genes are used to examine genetic heterogeneity and its implications in species
289 evolution (Lunt et al. 1996).

290 In this study, the generalist predator *Z. renardii* primers were designed based on the COI gene,
291 resulting in an amplicon of 100% coincidence in the tests conducted for this species. Moreover, the

292 primers of *D. citri*—the prey under study—obtained from the diagnostic protocol of *Candidatus*
293 Liberibacter spp. (DGSV 2010), generated a product 100% coincidence when compared the
294 sequence with NCBI and Boldsystems databases. It is worth mentioning that the primers of this
295 diagnostic protocol were designed based on the Wingless (Wg) gene, which codes for a diffusible
296 glycoprotein secreted by 26 species of psyllids (Thao et al. 2000). Despite the specificity obtained,
297 there was a reaction of the *D. citri* primers in the qPCR tests conducted on *Z. renardi* individuals
298 that were fed only *Drosophila* spp. (T5, laboratory experiment). Because of this result, a qPCR was
299 performed on samples of DNA from *Drosophila* spp. and *A. ludens*, the alternative species used in
300 the study. In both cases, positive responses to the *D. citri* primers were obtained, although the
301 threshold cycles (Ct) were different (Table 3). Likewise, a virtual PCR with GenBank database
302 gave more coincidence with *Drosophila* spp., the most used insects in our study. It should be
303 mentioned that in the experiments of this study, the Ct obtained in qPCR corresponded to *D. citri*.
304 Given that Wg is a gene present in insects that controls embryo development (Gonsalves and
305 DasGupta 2008) and formation of extremities in the adult stage (Schubiger et al. 2010), we believe
306 that the cross-linking observed in T5 may have been due to the presence of this gene in the DNA
307 of *Drosophila* spp. This problem can be avoided in the diagnostic protocol of *Candidatus*
308 Liberibacter spp. by using TaqMan probes, which were not used in this study because of its high
309 cost. Therefore, to avoid cross-linking in similar studies, it would be convenient to use TaqMan
310 probes, or even better, primers different from the Wg gene.

311 The variability observed among the laboratory experiment treatments could be explained by the
312 feeding habits of this predator. Cohen (1993) mentions that in *Z. renardii*, a sucking insect,
313 digestion begins with liquefying the prey before ingesting it. This is made possible by the
314 endopeptidase in its saliva. After ingestion, the material is subjected to more hydrolysis in the

315 midgut of the predator (Cohen 1993). Among the benefits of pre-oral digestion is an increase in
316 efficiency when ingesting a nutrient rich food (Cohen 1989) and a broader range of prey sizes that
317 the predator can consume (Cohen 1990).

318 Our results coincide with those reported by Cohen (1993), who states that *Z. renardii* digests a prey
319 of its own weight in little more than 48 h and is ready to consume another prey in less than 24 h
320 after the most recent feeding. We found that the half-life of *D. citri* DNA in the midgut of *Z.*
321 *renardii* was 20.8 h, while the time it takes to record the minimum detectable concentration of *D.*
322 *citri* DNA in the predator gut (0.16 ng/μL) was 42.1 h. However, the detection times estimated in
323 the laboratory do not necessarily reflect detection times under field conditions (Hoogendoorn and
324 Heimpel 2001) since in the field factors such as temperature, wind, rain and type of prey are highly
325 variable (Wiedenmann and O'Neil 1991; Parajulee et al. 1994). Moreover, the prey-predator
326 interactions are mediated by both density of the organisms and their habitat (Warfe and Barmuta
327 2004).

328 Greenstone et al. (2014) concluded that all sucking insects, and spiders, with which they share
329 metabolic adaptations, have a longer detectable prey DNA half-life in their gut than chewing
330 insects. Greenstone and Bennet (1980) also mention that sucking insects have lower basal
331 metabolic rates than other insects of comparable size. Likewise, in the field, predator insects
332 consume additional prey, which can cause longer periods of detection of target prey remains,
333 perhaps due to the reduction in rate of digestion (Hosseini et al. 2008). Because of this and our
334 results in the laboratory and cage experiments, it is necessary to allow *Z. renardii* more digestion
335 time in future studies. The results of our work also show that the proportion of target prey
336 consumed, relative to the alternative prey, does not affect the capacity of qPCR to detect the
337 presence of small quantities of digested *D. citri* DNA in the gut of *Z. renardii*; this minimum

338 detectable quantity was 0.16 ng/μL DNA. During the development of the experiments, it was
339 observed that *Z. renardii* fed on dead prey, both *D. citri* and the alternative prey. Given that feeding
340 on cadavers may have contributed to the wide variability observed in the results, we suggest
341 removing the dead prey from the experimental units, especially in the studies that use highly
342 sensitive molecular tests such as qPCR. According with our findings, qPCR is considered a useful
343 tool for the study of *D. citri-Z. renardii* interactions and for evaluating the potential of the assassin
344 bug as a natural enemy of the Asian citrus psyllid.

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464

465 Table 1. Effect of prey kind and post-feeding time on the concentration[1] of *D. citri* ADN in gut
466 content of *Z. renardii* adults fed with diferent combinations of adults of *D. citri* (DC) and
467 *Drosophila* spp. (DS), in the laboratory.

Time (h)	Treatments[2]					
	T1[3]	T2	T3	T4	T5	T6
8	$120.5^{bc}{}_{A}$	$98.8^{c}{}_{A}$	$70.9^{c}{}_{AB}$	$119.0^{bc}{}_{A}$	$195.6^{a}{}_{A}$	$168.4^{ab}{}_{A}$
12	$141.2^{ab}{}_{A}$	$109.3^{b}{}_{A}$	$35.8^{c}{}_{B}$	$90.3^{bc}{}_{AB}$	$190.0^{a}{}_{A}$	$179.4^{a}{}_{A}$
24	$112.6^{bc}{}_{A}$	$148.0^{ab}{}_{A}$	$103.2^{bc}{}_{A}$	$42.5^{d}{}_{BC}$	$194.2^{a}{}_{A}$	$53.8^{cd}{}_{B}$
48	$101.2^{bc}{}_{A}$	$129.3^{b}{}_{A}$	$58.7^{cd}{}_{AB}$	$26.7^{d}{}_{C}$	$214.3^{a}{}_{A}$	$131.0^{b}{}_{A}$

[1] Transformed data with the rank test. [2] Lowercase letters compare treatments (rows) and capital letters compare time (columns), according to comparison by orthogonal contrasts with Bonferroni correction (P<0.05). [3] T1, 30 DC+10 DS; T2, 20 DC+20 DS; T3, 10 DC+30 DS; T4, 40 DC+0 DS; T5, 0 DC+40 DS; y T6, 0 DC+0 DS.

468

469

Table 2. Effect of prey kind and post-feeding time on the concentration of *D. citri* ADN in gut content of *Z. renardii* adults fed with different combinations of adults of *D. citri* (DC) and *Anastrepha ludens* (AL), in cages.

Treatments		Concentration of *D. citri* ADN in gut content of *Z. renardii* adults (ng/µL) [2]
Number	Total number of prey added in four days [1]	
T1, Cage 1	640 DC + 0 AL	0.2263 b
T2, Cage 2	480 DC + 16 AL	0.2274 b
T3, Cage 3	320 DC + 32 AL	1.4940 a
T4, Cage 4	160 DC + 48 AL	0.1446 b

[1] T1, 160 DC per day; T2, 160 DC days 1-3 and 16 AL day 4; T3, 160 DC days 1-2 and 16 AL days 3-4; T4, 160 DC day 1 and 16 AL days 2-4. [2] The same letters indicate no statistical differences according to Tukey 5 %.

475 Table 3. Number of threshold cycles (Ct) in cross tests of qPCR with primers WGf and WGr with

476 DNA from the three prey and the predator used in the study.

Insect species	Threshold cycles (Ct)
Diaphorina citri (prey)	21.09
Drosophila spp. (prey)	36.45
Anastrepha ludens (prey)	34.68
Zelus renardii (predator)	36.28

477

478

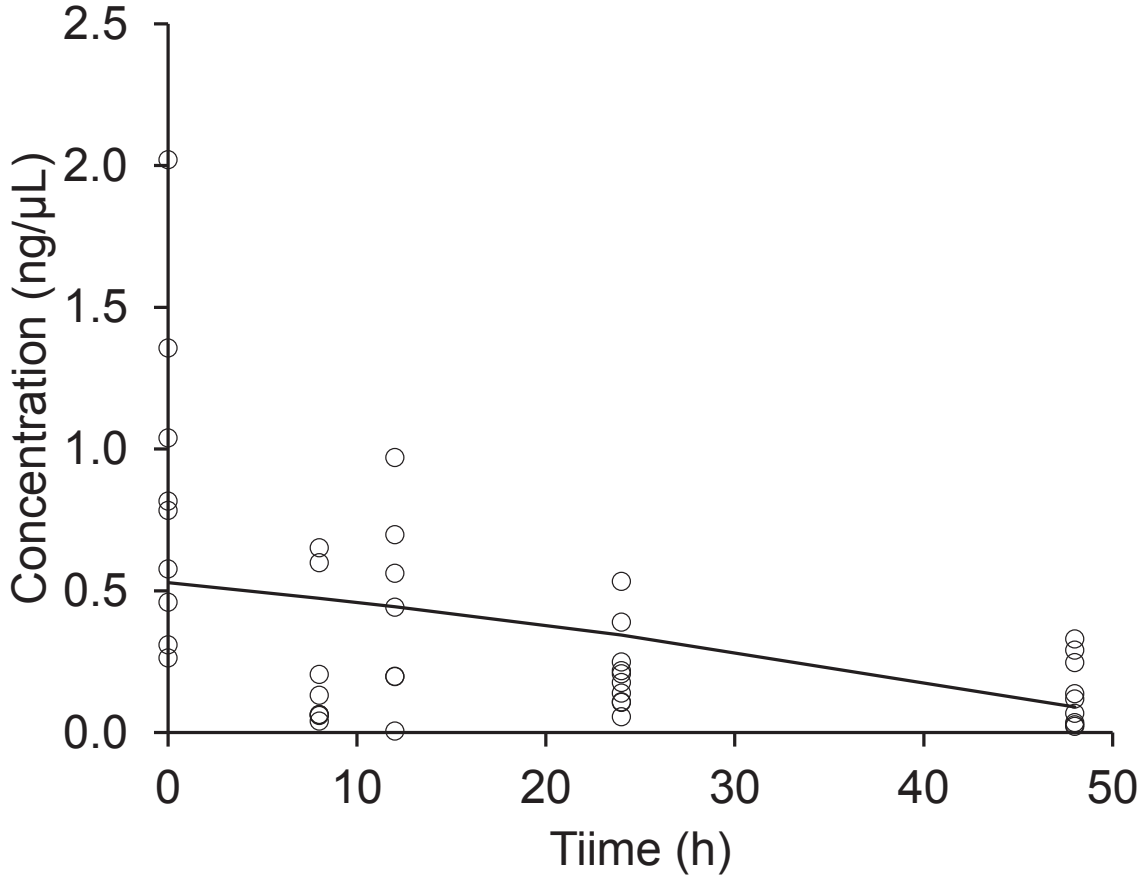
479 **Fig 1** Relationship between the concentration of *D. citri* ADN in gut content of *Z. renardii*

480 (*C*) and time (*t*) after prey were added, with equation $C = 1.02871 + (-0.5)\ e^{0.01312\ t}$

481 ($P>0.05$, $R^2= 0.20$)

482 Fig. 1

483



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Acknowledgements

We give special thanks to Enrique López-Pascasio (El Colegio de la Frontera Sur, ECOSUR) for the technical assistance in the laboratory; Javier Valle-Mora (ECOSUR) for advice in the statistical aspect of the study; and Dr. Allen C. Cohen (North Carolina State University) for advice in *Zelus renardii* gut dissections. We also thank the Programa Moscafrut (Secreataría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación – Instituto Interamericano de Cooperación para la Agricultura), particularly Dina Orozco, subdirector of production, for providing the *Anatrepha ludens* specimens used in this study. The study was funding in part by the SAGARPA-CONACYT project 2009-108591 "Management of the Huanglongbing (HLB) disease through control of populations of the vector *Diaphorina citri* (Hemiptera: Psyllidae), the Asian citrus psyllid." El Consejo Nacional de Ciencia y Tecnología (CONACYT, Mexico) provided a scholarship for Master of Sciences studies to CIAF (scholarship number 307954).

III. Conclusión

No obstante la gran variabilidad observada en los resultados y la presencia de algunas reacciones cruzadas, el análisis del contenido intestinal de *Z. renardii* mediante qPCR permitió estimar la vida media del ADN de *D. citri* en 20.8 h. Asimismo, el tiempo estimado para registrar la mínima concentración detectable del ADN de *D. citri* en el intestino del depredador (0.16 ng/µL) fue de 42.1 h.

Para evitar reacciones cruzadas en trabajos similares, se sugiere usar sondas TaqMan, o bien, emplear primers diferentes al gen Wg. También se sugiere remover las presas muertas de las unidades experimentales para evitar que los depredadores se alimenten de los cadáveres, y con ello reducir la variabilidad observada en los resultados.

La proporción consumida de presas blanco con respecto a las presas alternativas no afectó la eficacia de la qPCR para detectar la presencia de pequeñas cantidades de ADN de *D. citri* en el intestino de *Z. renardii*, por lo que se considera que la qPCR es una herramienta útil para estudiar la interacción *D. citri* - *Z. renardii* y evaluar el potencial de la chinche asesina como enemigo natural del psílido asiático de los cítricos.

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