ENDOCRINE CONTROL OF VITELLOGENESIS IN *BACTERICERA COCKERELLI* (HEMIPTERA: TRIOZIDAE), THE VECTOR OF 'ZEBRA CHIP'

A Dissertation

by

FREDDY ANIBAL IBANEZ-CARRASCO

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Cecilia Tamborindeguy
Committee Members,	Ginger Carney
	Patricia Pietrantonio
	Robert Coulson
Head of Department,	David Ragsdale

August 2017

Major Subject: Entomology

ABSTRACT

The potato psyllid, *Bactericera cockerelli* (Šulc), is a phloem-feeding insect with preference for *Solanaceae*. This insect species transmits the pathogenic bacteria '*Candidatus* Liberibacter solanacearum' (Lso) the causative agent of zebra chip, an important disease of commercial potatoes in several countries worldwide. The classification of psyllids among the most dangerous vectors has promoted their study, but still many biological processes need to be investigated. As a first step towards the elucidation of vitellogenesis in *B. cockerelli*, two candidate vitellogenin transcripts were identified and its expression was analyzed in different life stages. Our results showed that in virgin females, *BcVg1-like* expression increased up to 5 days old; while mating significantly upregulated its expression in 5- and 7-day-old females and also induced oviposition. *BcVg6-like* transcript was expressed at similar level between females and males and it was not up-regulated by mating. To elucidate the role of juvenile hormone in *B. cockerelli* Vgs expression, topical applications of juvenile hormone III (JH III) were performed on virgin females, resulting in an upregulation of *BcVg1-like* expression and an increase in the number of mature oocytes observed in female reproductive organs. While, *BcVg6-like* transcript did not showed changes in expression after exogenous application of JH III.

Presently five Lso-haplotypes have been identified worldwide; but only haplotypes A and B are associated with *Bactericera cockerelli* (Šulc.) in the Americas. Our results showed that no statistical differences in number of eggs oviposited and

developing oocytes between Lso-free and LsoA-infected females. In contrast, a significant reduction in number of eggs laid and developing oocytes were observed in LsoB-infected mated females. Expression analyses showed that *BcVg1-like* and *BcKr-h1* were highly expressed in Lso-free females after mating. A significant reduction in expression of *BcVg1-like* and *BcKr-h1* was observed in LsoB mated females. While no changes were observed in the expression of *S6 kinase*, *JH esterase* and *JH epoxide hydrolase* across different ages.

Overall, this study represents the first step to understand the vitellogenesis and interaction of Lso with *B. cockerelli*, highlighting the function JH III in the hormonal regulation and the role *BcVg1-like* and *BcKr-h1* in egg production and oocyte development.

DEDICATION

This dissertation is dedicated to my beloved wife Paula Castillo with who I had shared memorable experiences of my life, Paula as well gave me the indispensable love and strength to achieve our goals and always keep me on course. To my parents Rossana Miranda and Hector Ibanez who raised me with their love, support and encouragement through this journey. Also to my sister and brothers, Ada Ibanez, Tomas Ibanez and Hector Ibanez who reinforced and helped me to accomplish this goal.

I would also like to dedicate this dissertation in memory of my grandparents Armando Ibanez, Alicia Pinochet and Corina who passed away during this Ph.D., without their education and care during my childhood this achievement might never happened.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Cecilia Tamborindeguy, and my committee members, Dr. Ginger Carney, Dr. Patricia Pietrantonio and Dr. Robert Coulson, for their guidance and support during the course of this investigation, it was fun and challenging to have them in this committee, they always had different points of view that improved this investigation.

This achievement is because of two awesome persons that push me to take the first step, the GRE test, my wife Paula Castillo and friend Julien Levy. Also, my Ph.D. is because of my advisor, Dr. Cecilia Tamborindeguy, who not only provided the assistantship and funding; but, also advise me to take the best decisions in the academic and personal life. She is always a pleasure to talk and discuss ideas and cares about her graduate students and researchers, so they can succeed.

I also would like to thank Dr. Julian Rangel to included me in the Linnaean team and also to the department of entomology for let me be part of it and support our team (Adrian Fisher II, Pierre Lau, Carl Hjelmen and Derek Woller,), with who shared the first place as a team at Southwestern branch meeting.

All this research was not possible without my lab mates, the past and actual members thank you very much all of you, in special to Dr. Azucena Mendoza that was a great help with everything, being a great friend and a person that I will never forget. Also, she is an excellent professional providing the material, plants and insect colonies for this dissertation.

Thanks also go to Chloe Hawkings, Grayson Tsung and my friends Regina Mendoza and Denis Odokonyero who gave me the opportunity to share your friendship and stay in their homes, thank you for your hospitality and allowing us (Paula and me) to live in your lovely homes.

Thanks also go to all my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience. Finally, thanks to my mother and father for their encouragement and to my wife for her patience and love.

CONTRIBUTORS AND FUNDING SOURCES

This work was supervised by a dissertation committee consisting of Professors Cecilia Tamborindeguy, Patricia Pietrantonio and Robert Coulson of the Department of Entomology and Professor Ginger Carney of the Department of Biology.

All work for the dissertation was performed and completed independently by the student under the advisement of Cecilia Tamborindeguy of the Department of Entomology.

This work was made possible by National Institute of Food and Agriculture-Agriculture and Food Research Initiative (NIFA-AFRI) under the Grant Number 2012-67013-19431.

Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institute of Food and Agriculture and/or the Agriculture and Food Research Initiative.

TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

Page

Page

LIST OF TABLES

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Natural history of *Bactericera cockerelli* (Šulc)

With approximately 82,000 described species, Hemiptera is the fifth largest order of insects after Coleoptera, Diptera, Hymenoptera, and Lepidoptera (Arnett Jr, 2000, Cameron et al., 2006, Dolling, 1991). It consists of three main suborders, Sternorrhyncha (whiteflies, aphids, mealybug, and psyllids), Auchenorrhyncha (planthoppers, leafhoppers, treehoppers, spittlebugs and cicadas), and Heteroptera (seed bugs, stink bugs, assassin bugs, leaf-footed bugs and bedbugs) (Cryan and Urban, 2012). Many hemipterans have economic importance because they are pests of major agricultural crops (Wheeler, 2001, De Barro et al., 2011, Williams, 1996) and/or vectors of human, animal and plant pathogens (Dotson and Beard, 2001, Monteiro et al., 2003).

Bactericera cockerelli (Šulc) (Hemiptera: Triozidae), also known as potato psyllid or tomato psyllid, is a native species to North America. Presently, potato psyllids can be found not only in North America but also in Central America and New Zealand (Jackson et al., 2009, Munyaneza et al., 2009). This phloem feeding insect has a wide host range. While it prefers solanaceous plants, it can also complete its development on more than 40 host species from different plant families (Wallis, 1955). Due to its feeding behavior and capability to carry and transmit the plant pathogen '*Candidatus* Liberibacter solanacearum' (Lso), potato psyllids can damage several economically important solanaceous crops such as tomato, potato and pepper. In potato, the disease caused by Lso is called 'zebra chip'. The symptoms of the disease in raw tubers are

similar to a zebra hide pattern, thus the name, and include brown lesions of the vascular ring, necrotic flecking of internal tissues and occasionally streaking of the medullary ray tissues. This disease has a high economic impact on the potato industry (Munyaneza et al., 2007a, Munyaneza et al., 2007b), and therefore, an in depth understanding of the interactions between the pathogen and its vector is critical to manage the disease.

The potato psyllid life cycle is characterized by three life stages: egg, nymphal and adult (Figure 1.1). Eggs are orange-yellow, oblong in shape with an average length and width of 0.3 mm and 0.1 mm, respectively. The egg is attached to the leaf of the host plant with its 0.2 mm-long stalk. Potato psyllid nymphs go through five nymphal instars, and completion of nymphal development takes an average of 15.4 days (with a range of 12 to 44 days) (Yang and Liu, 2009, Nachappa et al., 2012a). After molting, teneral psyllid adults (newly emerged) can be of different colors such as pale green or light amber, becoming darker after three days. Adults' body length can vary from 1.3-1.9 mm and their lifespan can range from 16 to 97 days (Liu and Trumble, 2007). Both virgin and mated females can lay eggs after a preoviposition period that can vary from 3 to 25 days (Abdullah, 2008). Mating occurs several times during the female lifespan and mated females can oviposit on average more than 300 eggs during their lifetime (Yang and Liu, 2009).

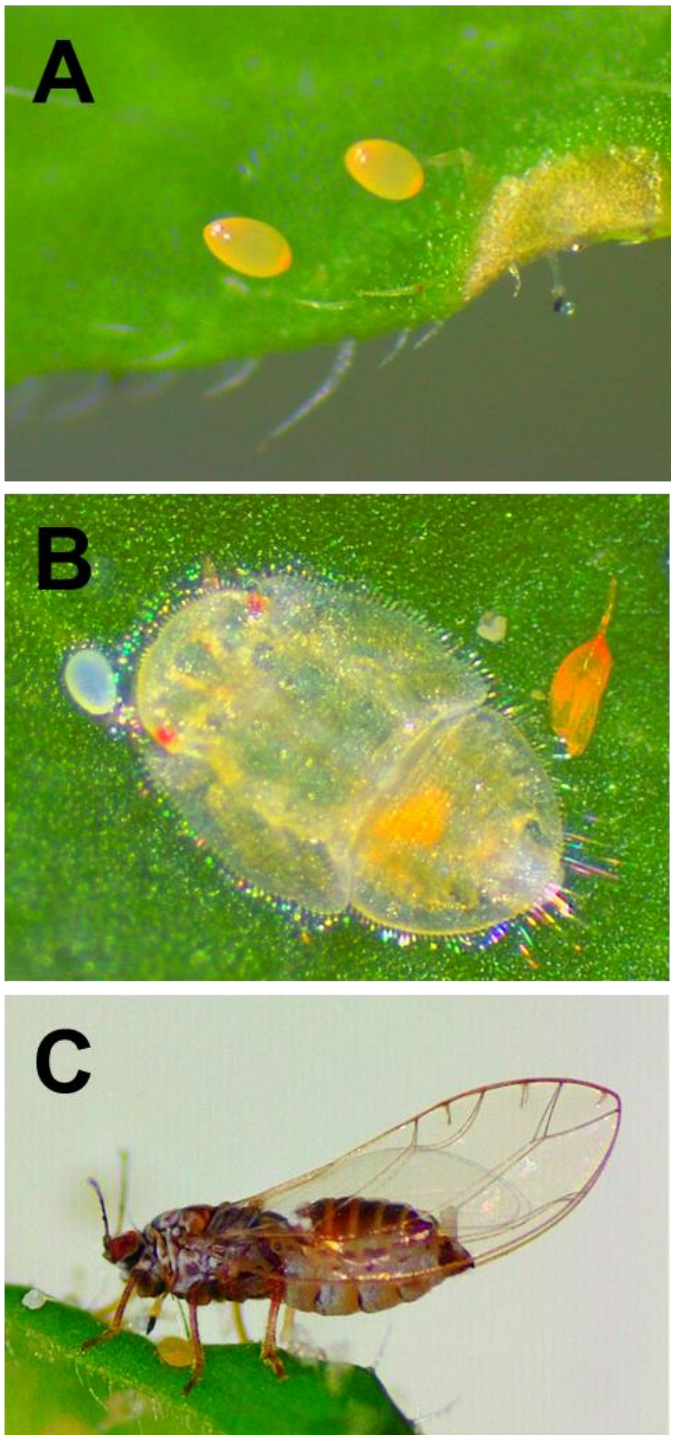

Figure 1.1. Life stages of potato psyllid, *Bactericera cockerelli* (Hemiptera: Triozidae). **A**, eggs, **B**, nymph and **C**, adult (female).

1.2 Vitellogenesis

Vitellogenesis is a complex biological process. In most insect species it involves a massive synthesis of the yolk proteins (i.e. vitellogenin) in the fat body, after which vitellogenin is deposited in the developing oocytes as vitellin by receptor-mediated endocytosis (Dhadialla et al., 1992). Insect vitellogenesis has been extensively studied in different insect species, particularly in members of the Diptera order such as flies and mosquitoes.

In the adult yellow fever mosquito female, *Aedes aegypti,* vitellogenesis can be divided into the **Previtellogenic period (preparation and arrest)** and the **Vitellogenic period (synthesis and termination)** (Figure 1.2). The previtellogenic period begins after eclosion of the adult female and is governed by juvenile hormone III. During the **Preparation** stage, which covers the first three days following female eclosion, the fat body and ovary tissues prepare the transcriptional and translational molecular machineries necessary for yolk protein precursor synthesis. This preparation stage is followed by the **Arrest** stage, during which the formation of Ecdysone receptor/Ultraspiracle (EcR/USP) complex is prevented and therefore the 20-hydroxyecdysone-dependent transactivation is blocked. The arrest stage lasts until the female has a blood meal, which triggers the vitellogenic period. The vitellogenic period begins with a **Synthesis** stage which lasts between 0 and 30 hours. During this stage, the active form of ecdysteroids, 20-hydroxyecdysone (20E), binds to the EcR/USP complex in the fat body tissue and begins the transcription and synthesis of yolk protein precursors (YPPs), such as vitellogenins. These proteins are then secreted into the

hemolymph and internalized via receptor-mediated endocytosis into the oocytes. Finally, during the **Termination** stage, the synthesis of YPPs is stopped and the fat body cells enter a process of remodeling called "autophagy" that permits a new vitellogenic cycle (Zhu et al., 2000, Raikhel et al., 2002, Zhu et al., 2003).

However, knowledge of the vitellogenic process and its endocrine control in phloem feeding hemipterans is very limited, and improving the information available might be critical to develop better strategies of integrated pest management.

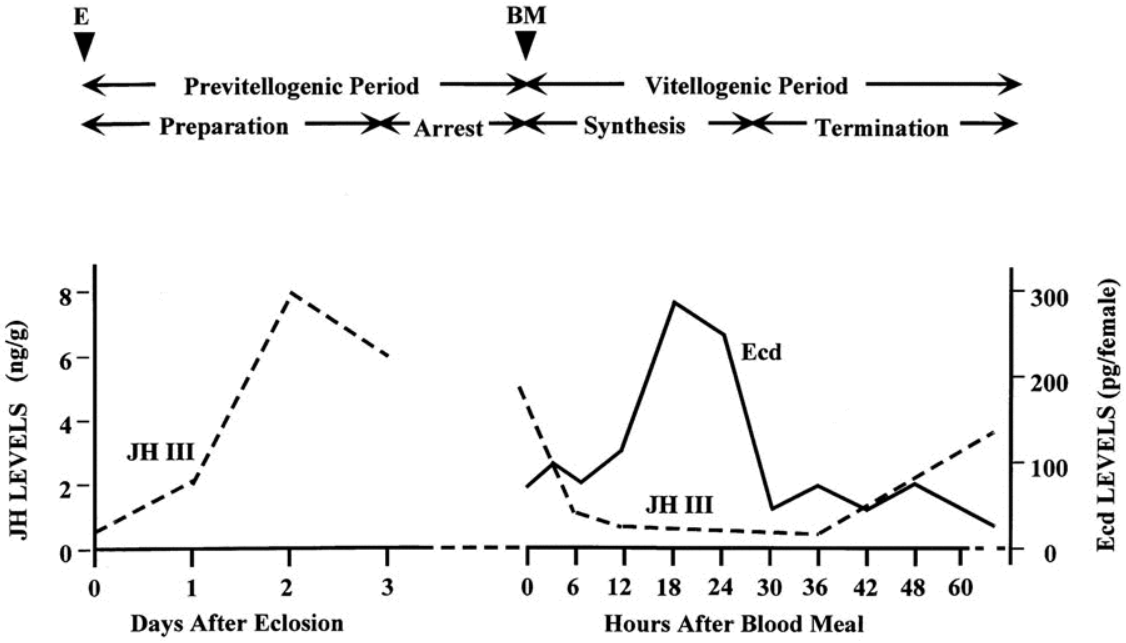

Figure 1.2. It was obtained from the manuscript published by Zhu et. al., 2000. AHR38, a homolog of NGFI-B, inhibits formation of the functional ecdysteroid receptor in the mosquito Aedes aegypti. The EMBO journal, 19, 253-262

Figure 1.2. Hormonal titers observed during the first vitellogenic cycle in the adult yellow fever mosquito female, *Aedes aegypti*. BM, blood meal; E, eclosion; JH III, juvenile hormone III titers; and Ecd, ecdysteroid titers. This figure was obtained from manuscript previously published (Zhu et al., 2000).

1.3 Insect vitellogenins

Vitellogenins (Vgs) are large proteins (Tufail and Takeda, 2008). Many insect species have only one Vg gene; however, some species have multiple Vg genes, see review (Tufail et al., 2014). For example, the Madeira cockroach *Leucophaea maderae* has two Vg genes (Vg1 and Vg2) that produce proteins with 96% similarity at the amino acid level (Tufail et al., 2007). Why some insects possess multiple Vg genes is still unclear. Recently, it was suggested that different Vg genes might aid in the regulation of hormonal dynamics and the coordination of social organization of worker and male honey bees (Guidugli et al., 2005, Nelson et al., 2007).

Vitellogenin mRNAs (transcripts) are 6 to 7 kilobase pairs (kbp) long (Tufail and Takeda, 2008) and encode a primary protein precursor of ~200 kilo Dalton (kDa) that is homologous to phosphoglycolipoproteins (Tufail et al., 2005). Based on amino acid sequence comparisons, it was determined that the Vg protein is composed of 5 subdomains with high amino acid conservation. Features such as polyserine regions, cleavage site, GL/ICG and DGXR motifs can be found on these subdomains (Lee et al., 2000b). Except in members of the suborder Apocrita in hymenopterans and depending on the insect species, insect Vgs have one or more cleavage site motifs (R/KXXR/K). These cleavage site motifs are recognized and proteolytically cleaved by the subtilisin-like endoproteases (e.g. convertases) before secretion, resulting in a large 140–190 kDa and a small 40-60 kDa subunit (Rouillé et al., 1995). This motif is commonly found near to the N-terminal and can be flanked by polyserine region(s) (Tufail et al., 2014). The polyserine region(s) might serve as phosphorylation sites, as shown in the cockroaches

Periplaneta americana and *L. maderae* (Tufail et al., 2001, Tufail et al., 2005, Tufail and Takeda, 2002). It has been proposed that the negative charge generated by high concentrations of phosphoserines in Vg might increase the protein solubility or Ca^{2+} and Fe^{3+} chelation (Gerber-Huber et al., 1987, Goulas et al., 1996). In *Aedes aegypti*, Vg dephosphorylation has been associated with a reduction in binding between Vg and its receptor (VgR) indicating that phosphate moieties (phosphoserines) are required for a proper Vg uptake into the oocytes (Dhadialla et al., 1992).

Several motifs are commonly found in the C-terminal region of insect Vgs (Figure 1.3) including, DGXR motif, GL/ICG motif and cysteine residues in conserved positions (Tufail et al., 2005, Mouchel et al., 1996). The DGXR motif is located 17-19 residues upstream of the GL/ICG motif in all insect Vgs, except in *L. maderae* (Tufail et al., 2001). *Nilaparvata lugens*, *Homoladisca coagulata* and *Solenopsis invicta* Vgs lack the arginine (R) in the DGXR motif. In vertebrates the GL/ICG motif and cysteine residues are necessary for a correct oligomerization of vitellins, the processed vitellogenin protein in the egg (Mouchel et al., 1996). In addition, it has been proposed that the amino acids DG from the DGXR motif together with the GL/ICG motif and cysteine residues at conserved positions might form a structure necessary for vitellins to function properly (crystallization of vitellins) during embryogenesis (Tufail et al., 2001).

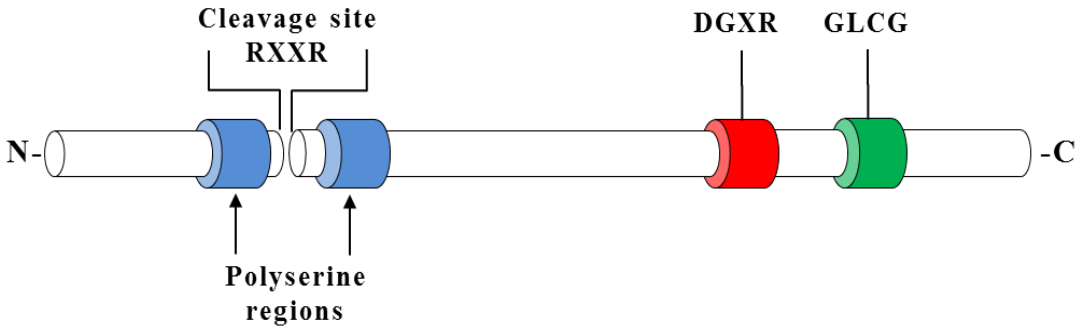

Figure 1.3. Schematic representation of the motifs commonly found in insect Vitellogenins. RXXR sequence (putative or determined) is a consensus cleavage site used during post-translational proteolytic processing of vitellogenins. Blue cylinders indicate the polyserine regions, red cylinder is shown the DGXR motif and in green cylinder is indicated the GL/ICG motif.

1.4 Endocrine control of insect vitellogenesis

Multiple reproductive strategies have evolved among the diverse class Insecta, and depending on the insect species, vitellogenesis is predominantly controlled by different factors including neuropeptides, juvenile hormones (JHs) and/or ecdysteroids (Raikhel et al., 2005). Juvenile hormones are a family of acyclic sesquiterpenoids synthetized and secreted by the corpora allata glands (Wigglesworth, 1970). Several JH forms have been identified in insects species, these molecules are: JH 0, JH I, JH II, JH III, 4-methy JH I, JH bisepoxide$_3$, methyl farnesoate, and JH skipped B$_3$ (Sehnal, 1984, Noriega, 2014). Among them, JH III (methyl (2E,6E)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate) is the most common JH form involved in Vg upregulation (Tufail et al., 2014). Other important functions of JH in insects include regulation of metamorphosis,

caste determination, diapause, and polyphenisms (Raikhel et al., 2005, Riddiford, 1994, Hartfelder and Emlen, 2012).

Ecdysteroids represent a family of sterol derivatives containing more than 300 members that share common structural features; the active form of insect ecdysteroids is 20-hydroxyecdysone (Lafont et al., 2012). In immature stages of insects, ecdysteroids are secreted by the prothoracic glands and their main function is the regulation of important genes that control ecdysis and metamorphosis. In adults, ecdysteroids play an important role in the control of reproduction and depending of the insect species, these hormones can be synthesized in the ovaries (Rees et al., 2010), in testes (Loeb et al., 2001, Vafopoulou and Steel, 2005) and/or in the abdominal integument (Delbecque et al., 1990, Spindler and Spindler-Barth, 1991).

Juvenile hormone and 20-hydroxyecdysone are the important hormones involved in the regulation of insect Vg transcript expression and protein synthesis (Weaver and Edwards, 1990, Wyatt and Davey, 1996, Raikhel et al., 2002). Based on the hormonal regulation of Vg transcription, insects are categorized in three major groups. The first group includes the majority of insect species in which Vg transcription is regulated only by JH (Belles, 1998, Engelmann, 1983); the second group includes insect species such mosquitoes (Diptera), in which Vg regulation requires JH and then ecdysteroids (Hagedorn et al., 1975, Raikhel et al., 2005); and the third group includes lepidopterans and other insects that require both JH and ecdysteroids (together) for Vg regulation (Belles, 1998).

In the first group, cockroaches (Dictyoptera) are the classical model for studying JH-dependent vitellogenesis. For example, in the American cockroach *Periplaneta americana* the synthesis and uptake of Vg, oocyte growth and ootheca formation is blocked in allatectomized females or females treated with JH inhibitors, while applications of exogenous JH can restore these processes (Weaver and Edwards, 1990). Also, molecular evidence in *P. americana* showed that the transcription of both Vg genes (Vg1 and Vg2) was activated by exogenous applications of JH III (Tufail et al., 2001). In the German roach, *Blattella germanica*, Vg synthesis and cyclic maturation of oocytes depends on JH III (Belles, 1998), as shown using cardio-allatectomized females treated with JH III (Comas et al., 1999). Similarly, in *L. maderae*, Vg synthesis is controlled by JH or its analogue methoprene in adult females and males as well as in last-instar nymphs of both sexes (Don-Wheeler and Engelmann, 1991). Vg induction by JH and/or its analogs has been reported for other cockroach species including *Nauphoeta cinerea* and the Pacific beetle cockroach *Diploptera punctata*. (Don-Wheeler and Engelmann, 1991).

JH is also the principal hormone governing Vg synthesis in hemipterans. Most of the studies have been performed in the kissing bug *Rhodnius prolixus*, the vector of 'Chagas disease', in which JH is responsible for Vg synthesis in the fat body (Davey, 1993). Similar regulation occurs in other hemipteran heteropteran species including hematophagous bugs such as *Triatoma protracta* (Uhler) and phytophagous insects such as *Oncopeltus fasciatus* and *Pyrrhocoris apterus* (L.) (Engelmann, 1983). In hemipterans, such as the giant water bug, *Lethocerus deyrollei*, Vg transcript expression

in females was induced upon JH III injection and inhibited when they were exposed to 20-hydroxyecdysone (20E), suggesting that the Vg gene was regulated by both hormones (Nagaba et al., 2010). Similarly, in the two wing-morphs of the brown planthopper *Nilaparvata lugens,* topical application of JH III upregulated Vg transcript expression (Tufail et al., 2010). Presently, only one Vg gene has been characterized in phloem feeding *Sternorrhyncha*. Gene expression analysis showed that *Bemisia tabaci* Vg mRNA was first detected during the red eye pupal stage and the highest expression was observed in adults six days after eclosion. While the Vg protein was also detected in the red eye pupal stage, there was a rapid increase in the protein level between 6 and 12 days after eclosion, followed by a reduction in the Vg protein abundance until the insect's death (Guo et al., 2012).

The second type of hormonal control of vitellogenesis has been extensively studied in mosquitoes and flies (Diptera). In this group, the regulation of reproduction involves JH and ecdysteroids. For example, in the anautogenous mosquito *Aedes aegypti,* JH is important for Vg production, because this hormone capacitates the fat body during the previtellogenic phase to have the proteins necessary for the action of 20E (Dhadialla and Raikhel, 1994, Dhadialla et al., 1992, Tufail et al., 2014). In addition, ovaries can synthetize and secrete the inactive factor, ecdysone, which is converted into the active form of the hormone, 20-hydroxyecdysone required for Vg protein synthesis by the fat body (Hagedorn et al., 1975).

In the third group, the reproductive regulation varies among insect species. In the lepidopterans *Hyalophora cecropia* and *Bombyx mori*, the Vg synthesis begins in the

early pupal stages and is regulated by 20E (Tsuchida et al., 1987). In *Lymantria dispar* (L.) vitellogenesis begins in late instar larvae and topical application of JH inhibits Vg transcript expression, suggesting that a declining or low JH titer are crucial for Vg induction in this species (Fescemyer et al., 1992). However, in *Spodoptera frugiperda*, the expression of the Vg is regulated by both hormones, JH and ecdysteroids; and the Vg uptake by oocytes seems to be controlled only by JH (Sorge et al., 2000).

Neuropeptides also play an important role in the regulation of vitellogenesis. Some of them are allatostatin (AST) and allatotropin (AT), both regulate the synthesis and release of JH. The neuropeptide AT induces the JH synthesis and release by the corpora allata (Kataoka et al., 1989, Hernández-Martínez et al., 2007), while AST rapidly and reversibly inhibit JH synthesis (Stay and Tobe, 2007).

1.5 Research Significance

As summarized above, vitellogenesis in insects is a finely regulated process under the control of JH and/or 20E, which can act together or alone. Also, the reproductive success of all oviparous species depends on Vg biosynthesis and its accumulation in the developing oocytes as vitellin. Therefore, the questions addressed in this study will include identifying the reproductive phases in *Bactericera cockerelli* females, determining the Vg transcript and protein expression profile, and clarifying or defining the main hormone involved in *B. cockerelli* vitellogenesis. Finally, the information and knowledge generated in this work will help in the identification of putative targets involved in vitellogenesis to generate new methods to control psyllid populations, which have emerged as major pests of crops in the United States and elsewhere. We also expect that the information might be applied to other phloem feeding hemipterans for which very little is known.

2. IDENTIFICATION AND EXPRESSION ANALYSES OF VITELLOGENIN IN *BACTERICERA COCKERELLI* (ŠULC)[1]

2.1 Overview

The potato psyllid, *Bactericera cockerelli* (Šulc) (Hemiptera: Triozidae), is a phloem-feeding insect with preference for *Solanaceae*. This insect species is vector of the pathogenic bacteria '*Candidatus* Liberibacter solanacearum' the causative agent of zebra chip, an important disease of commercial potatoes in several countries worldwide. The recent classification of psyllids among the most dangerous vectors has promoted their study, but still many biological processes such as reproduction and vitellogenesis need to be investigated. As a first step towards the elucidation of vitellogenesis in *B. cockerelli*, one candidate vitellogenin transcript (6,622 bases long) was identified and its expression was analyzed in virgin and mated females between 1 and 7 days old. Our results showed that in virgin females Vg expression increased up to 5 days old; while mating significantly up-regulated Vg expression in 5- and 7-day-old females and also induced oviposition. To determine the role of juvenile hormone in *B. cockerelli* Vg expression, topical applications of juvenile hormone III were performed on virgin females, resulting in an up-regulation of Vg expression and an increase in the number of mature oocytes observed in female reproductive organs. Overall, this study represents

[1] Reprinted with permission from Identification and expression analyses of vitellogenin in *Bactericera cockerelli* (Šulc), Freddy Ibanez, Julien Levy and Cecilia Tamborindeguy, 2017. Journal of Insect Physiology, 98, 205-213. Copyright 2017 by Elsevier.

the first step to understand vitellogenesis of *B. cockerelli* and it highlights the role of JH III in the hormonal regulation of Vg expression and oocyte development.

2.2 Introduction

Psyllids are phloem feeding hemipterans that have emerged as major vectors of plant pathogenic bacteria. In particular, they vector '*Candidatus* Liberibacter spp' that are threatening several crops' production worldwide (Aubert, 1992, Bové, 2006, Secor et al., 2009, Manjunath et al., 2008). Presently, no effective resistance against any *Liberibacter* bacterium has been deployed in any crop system. Therefore, disease control relies on chemical control of the psyllid vector. In spite of psyllids' importance as vectors, very little is known about psyllid physiology, in particular reproduction. Understanding the processes involved in psyllid reproduction could help us develop more efficient and comprehensive control strategies.

The reproductive success of all oviparous species, including insects, relies on vitellogenin (Vg) biosynthesis and its accumulation, as vitellin, in the developing oocytes (Raikhel, 1992). In most insects, Vgs are synthesized by the female fat body cells, but in others like in Cyclorrhapha, Vgs are also synthetized by the ovarian follicle cells as a complementary vitellogenic organ (Belles, 1998). From a biochemical and molecular perspective, Vgs are high molecular weight protein precursors (~200 kDa), encoded from a 6 to 7 kilobases (kb) transcript (Tufail and Takeda, 2008, Tufail et al., 2014). Common features of the Vg amino acid sequence include polyserine regions, cleavage sites, and GL/ICG and DGXR motifs (Lee et al., 2000b). In most insects

species, Vgs possess one or more cleavage site motifs (R/KXXR/K), and are proteolytically processed by subtilisin-like endoproteases, resulting in most insects in a large 140–190 kDa and a small 40-60 kDa subunits, which are assembled together and then secreted into the hemolymph as big oligomeric phosphoglycolipoproteins (400–600 kDa) (Tufail et al., 2014). Near the cleavage site(s), polyserine regions can be found commonly (Rouillé et al., 1995), for which several functions have been proposed such as phosphorylation sites (Tufail et al., 2001, Tufail et al., 2005, Tufail and Takeda, 2002), solubility, and cations (Ca^{2+} and Fe^{3+}) chelation (Gerber-Huber et al., 1987, Goulas et al., 1996). In *Aedes aegypti*, the dephosphorylation of these regions has been associated with an impairment in binding to the vitellogenin receptor, indicating that the phosphoserines might be required for the Vg uptake into the oocytes (Dhadialla et al., 1992). Common features of the C-terminal region of insect Vgs include the GL/ICG and DGXR motifs and cysteine residues in conserved positions (Tufail et al., 2005, Mouchel et al., 1996). A DGXR motif is present upstream of the GL/ICG motif in all insect Vgs except in *Leucophaea maderae* (Tufail et al., 2001). In vertebrates the GL/ICG motif and cysteine residues are necessary for a proper oligomerization of vitellin in the oocyte (Mouchel et al., 1996).

The control of insect vitellogenesis depends on the coordinate action of different factors including neuropeptides, juvenile hormone (JH) and/or ecdysteroids (Raikhel et al., 2002, Wyatt and Davey, 1996, Tufail et al., 2005). Juvenile hormone are a family of acyclic sesquiterpenoids synthetized and secreted by the corpora allata glands (Wigglesworth, 1970). Juvenile hormone is involved in the control of Vg transcription in

most insect species (Tufail and Takeda, 2008), in particular in members of the Hemiptera order, such as *Nilaparvata lugens*, *Lethocerus deyrollei* and *Riptortus clavatus* (Shinoda et al., 1996, Tufail et al., 2010, Nagaba et al., 2011).

The goals of this study were (a) to identify Vg in the potato psyllid, *Bactericera cockerelli;*(b) to analyze its expression profile; and (c) to determine whether topical applications of JH III affect Vg transcript and protein expression.

2.3 Materials and methods

2.3.1 Insects

The *B. cockerelli* colony northwestern haplotype used for this study was obtained in 2013 from Dr. Henne, AgriLife Research Weslaco, TX. The colony was maintained on tomato plants in 14'' X 14'' X 24" insect cages (BioQuip, Rancho Dominguez, CA, USA) at room temperature and photoperiod of 16:8 h (Light: Dark).

To obtain adult cohorts of specific age, 4^{th}-5^{th} instar nymphs were transferred from the colony into different 4-week-old tomato plants. On the day adults emerged, they were sexed under a dissection microscope (Olympus SZ-ST, Japan) and females and males were transferred separately to new plants. At the desired age (1-, 3-, 5- and 7-days-old), samples were flash frozen in liquid nitrogen and stored at -80º C for further analysis. To obtain 5- and 7-day-old mated females, 3-day-old virgin females and males were placed into a 1.7 mL Eppendorf tube for copulation during a period of 2 to 3 hours after which only mated females were transferred to new plants for further collection and analysis as was previously described.

2.3.2 Sequence identification and cloning

Bactericera cockerelli transcriptomic dataset was (Nachappa et al., 2012a) datamined to identify putative Vg sequences using BLASTX. Specific primers (Table 2.1) were designed with Primer 3 web (Untergasser et al., 2012) to validate the *in silico* Vg contig predictions, link non-overlapping Vg contigs, and to determine the 3'-cDNA extremity sequence using RACE-PCR since it was not represented in the existing contigs.

Total RNA from thirty *B. cockerelli* adults (15 females and 15 males) was purified using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions and five hundred ng of total RNA was used for cDNA synthesis using Verso cDNA Synthesis kit (Thermo, Waltham, MA) plus anchored-Oligo (dT) primers following the manufacturer's instructions, then the cDNA samples were stored at -20º C until further use. To validate and link non-overlapping Vg contigs, RT-PCR reactions were performed using PrimeSTAR Max DNA Polymerase (Clontech, Mountain View, CA). Each PCR reaction contained 25 ng of cDNA, 150 nM of each specific primer and 1x of PrimeSTAR Max Premix; the volume was adjusted with nuclease-free water to 50 µL. The PCR conditions were 95°C for 2 min; followed by 35 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30-90 sec (depending of amplicon size); and a final extension at 72°C for 5 min. Amplicons were visualized in a 0.8% agarose gel, and amplicons of the expected size were excised from gel and purified using PureLink Quick Gel Extraction kit (Invitrogen, Carlsbad, CA). Each PCR fragment (150 ng) was cloned in pGEM-T easy vector using the pGEM-T Easy cloning kit (Promega, Madison, WI)

and transformed into One Shot TOP10 competent cells (Invitrogen, Carlsbad, CA). For each construct, plasmid DNA from at least three colonies was purified using PureLink Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad, CA) and sequenced by Eton Bioscience Inc (San Diego, CA, USA). Obtained sequences were compared to *B. cockerelli* transcriptomic data to verify sequence predictions.

To obtain the full-length Vg transcript, 1 µg of total RNA from thirty *B. cockerelli* adults (15 females and 15 males) was used as template for synthesis of 3' Rapid Amplification of cDNA Ends (RACE) library (Takara Bio USA, Madison, WI) following the manufacturer's instructions. RACE-PCR reactions were performed in a final volume of 50 µL, containing 50 ng of the first-strand cDNA (3' RACE cDNA) reaction as a template, 5 µL of 10x Advantage 2 PCR buffer, 2 µL of 10 mM dNTPs, 2.5 µL (10 mM) of Vg-specific primer (BcVg1-like FL Fw1, Table 2.1), 4 µL of Universal Primer A Mix (UPM; Takara Bio USA, Madison, WI), 1 U of 50x Advantage 2 polymerase mix (Takara Bio USA, Madison, WI) and molecular grade water (Bioscience, VWR). The touchdown PCR conditions were 94°C for 2 min; followed by 5 cycles of 94°C for 30 sec, 68°C for 6 min and then 30 cycles of 94°C for 30 sec, 64°C for 30 sec, and 72°C for 6 min; and a final extension at 72°C for 10 min. Vg amplicon was gel-purified, cloned and then sequenced with several primers (Table 2.1) as was previously described.

Table 2.1. Primers used for *Bactericera cockerelli vitellogenin* candidate identification and gene expression analyses.

Primers		
Purpose	**Name**	**Sequence**
Link contigs	BcVg1-like 42586-47344Fw1	5-CCGACTTTTCCCCAAGAAAG-3
	BcVg1-like 42586-47344Rv1	5-TTGACGGCTGAGTTGACTTG-3
	BcVg1-like 45015-41973Fw1	5-GCGTAACGCTGACAAAAACA-3
	BcVg1-like 45015-41973Rv1	5-CGGCTTGAGCATCTTTGATT-3
RACE PCR	BcVg1-like FL Fw1	5-CCGACCGATTCAGTCATTCAG-3
Expression analysis	BcVg1-like qPCR Fw1	5-GACCTGTTGGGACTTTTGGA-3
	BcVg1-like qPCR Rv1	5-GCTTGTTTGGCGTTCTTCTC-3

2.3.3 Bioinformatics and phylogenetic analyses

Full length Vg sequence was *in silico* translated using the Open Reading Frame Finder tool (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). The predicted amino acid sequence was analyzed using the NCBI Conserved Domain Database search (Marchler-Bauer et al., 2014).

For the phylogenetic analysis, *B. cockerelli* Vg sequence was aligned with other 56 animal Vgs using ClustalW with MEGA 5.2 software (Tamura et al., 2011). The phylogenetic relationships of vitellogenins were assessed using Bayesian inference ran in MrBayes 3.2 software (Huelsenbeck and Ronquist, 2001) with the following parameters: four chains, two runs, amino acid model = GTR (Wag), rate variation = "invgamma" (GTR+I+Gamma model), and the analysis of Metropolis-coupled Markov chain Monte Carlo (MCMC) run was six million generations, sampled every 1000th step, and the first 25% of sampled trees were discarded as burn-in. The runs were considered converged when average standard deviation was lower than 0.01 and potential scale reduction factor value (PSRF) approached 1.0. The values of branch support were obtained by the method of posterior probability (≥0.75). The tree was rooted at midpoint, and saved and edited by Figtree program v.1.4.0 (Rambaut, 2012).

2.3.4 Gene expression analyses by RT-qPCR

Samples of total RNA were isolated from pools of 10 insects per biological replicate homogenized for 1 minute on ice with a plastic pestle using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Genomic DNA

contamination was eliminated by DNase treatment with Turbo DNase (Ambion, Invitrogen, CA). Total RNA quantity and purity was determined using an Infinite® 200 PRO NanoQuant (Tecan, Männedorf, Switzerland) and RNA integrity was visualized by electrophoresis in agarose gels at 1.2% stained with ethidium bromide. Three hundred ng of total RNA were used as template for cDNA synthesis as described previously.

For gene expression analyses, RT-qPCR reactions were performed using SensiFAST SYBR Hi-ROX Kit (Bioline, Taunton, MA) according to manufacturer's instructions. Each reaction contained 5 ng of cDNA, 250 nM of each primer (Table 2.1) and 1x of SYBR Green Master Mix; the volume was adjusted with nuclease-free water to 10 µL. The real-time PCR program was 95°C for 2 min followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec. Primers were designed using Primer3 web (Untergasser et al., 2012). Real-time PCR assays were performed using an Applied Biosystems ABI 7300 real-time PCR Thermocycler (Applied Biosystems). Reactions for all samples were performed in triplicates with a negative control in each run. The threshold cycle (Ct) values and the efficiency of each primer set for RT-qPCR were determined using LinRegPCR software (Ramakers et al., 2003). Primer specificity was monitored with melting curve analysis using Sequence detection system (SDS) version 1.4.0.27 (Applied Biosystems) and 2% agarose gel electrophoresis. The relative expression of the vitellogenin gene was estimated with the delta delta CT method (Schmittgen and Livak, 2008), using two reference genes *elongation factor-1a* (GenBank KT185020) and *ribosomal protein subunit 18* (GenBank KT279693) (Ibanez and Tamborindeguy, 2016).

2.3.5 Protein expression analyses

Protein samples were extracted from eggs, nymphs, and 1-, 3-, 5- and 7-day-old males and females. Briefly, ten individuals or a minimum of 100 eggs were placed in a 1.7 mL Eppendorf tube and homogenized on ice with a plastic pestle using 100 µL of 1x RIPA buffer (Invitrogen, Carlsbad, CA) supplemented with protease inhibitor tablets (Mini, EDTA-free, EASY pack, Roche Diagnostics, IN, USA). Homogenized samples were incubated for 30 minutes on ice and then centrifuged at 14,000g for 15 minutes, the supernatant was transferred to a new tube and each sample was quantified by Bradford protein assay using Coomasie protein assay reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Thirty µg of each protein extract were loaded in gradient NuPAGE® Novex® 4-12% Bis-Tris (Invitrogen, Carlsbad, CA) gels and stained with SimplyBlue™ SafeStain (Invitrogen, Carlsbad, CA). Finally, the bands corresponding to Vg candidate protein were analyzed and identified by LC/MS/MS in TAMU facilities.

2.3.6 Juvenile hormone III topical treatment

Juvenile hormone III (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in a solution of acetone/ethanol (ratio 80:20). For each replica, fresh solutions of JH III were prepared from the stock, at four different concentrations (0.002, 0.02, 0.2 and 2.0 µg/µL), and 0.5 µL of the corresponding solution was topically applied to the abdomen of 3-day-old virgin females (final doses of 0.001, 0.01, 0.1 and 1.0 µg/insect, respectively) held with a vacuum system. After topical applications, treated females

were transferred to 4-week-old tomato plants and four analyses (survival, oviposition, number of mature oocytes, and vitellogenin transcript and protein expression) were conducted in triplicates using 10 virgin females per replicate, as follows:

2.3.6.1 *Survival*

The survival of virgin females after treatment was recorded each 24 h for a 96-h period.

2.3.6.2 *Oviposition*

The effect of JH III in oviposition was examined by counting the number of eggs oviposited by 10 virgin females per replicate on tomato plants 96 h post JH III treatment.

2.3.6.3 *Number of mature oocytes*

To assess the effect of JH III in oocyte maturation, reproductive organs were dissected after 96 h post-treatment as described in (Ibanez et al., 2014). The images were obtained using an Axioimager A1 microscope (Carl Zeiss microimaging, Thornwood, NY, USA) and visualized with Axiovision Rel 4.8 software (Carl Zeiss).

2.3.6.4 *Vitellogenin transcript and protein expression*

The effect of JH III on Vg expression was assessed by RT-qPCR as described above. Briefly, ten 3-day-old virgin females were treated with 0, 0.01 or 1.0 µg of JH III per insect, and after 15 h the relative expression of Vg gene transcript was determined.

The protein abundance was assessed after 96 h post JH III treatment with one of the following doses 0, 0.001, 0.01, 0.1 and 1.0 µg/insect.

2.3.7 Statistical analyses

The effect of juvenile hormone III in oviposition and number of mature oocytes, and quantitative PCR analyses were analyzed using one-way ANOVA with Tukey's post hoc test in JMP 12.0 software. Survival analysis was performed using Kaplan-Meier method and differences between treatments were investigated by the log-rank test using SPSS software.

2.4 Results

2.4.1 Identification of *Bactericera cockerelli vitellogenin*

Datamining of *B. cockerelli* transcriptome resources using the BLASTX program resulted in the identification of 6 contigs showing similarities to insect vitellogenin proteins. These contigs were named as following: contig 42586 (2,649 bp), contig 47344 (398 bp), contig 45015 (618 bp), contig 41973 (1,507 bp), contig 29195 (286 bp) and contig 42587 (827 bp). Specific primers (Table 2.1) were designed to verify the bioinformatics prediction and also to link these contigs by PCR. To obtain the complete cDNA encoding for a putative Vg in *B. cockerelli*, RACE-PCR was performed which yielded a complete transcript sequence of 6,622 bases with an open reading frame (ORF) of 6,345 bases long. This candidate Vg was annotated as BcVg1-like (GenBank number KX752432) and named based on its similarity to another insect vitellogenins.

2.4.2 Structural analysis of BcVg1-like protein

In silico translation resulted in an open-reading frame of 6,345 bases encoding a 2,114 amino acids long protein. Bioinformatics analysis of the BcVg1-like amino acid sequence by NCBI Conserved Domain Database search (Marchler-Bauer et al., 2014) predicted two large domains. The first domain consisted in a vitellogenin-N domain, also known as the lipoprotein N-terminal, at amino acid position 609–951 (E-value = 1.25e-93). The second domain was a Von Willebrand factor type D (VWD) domain identified in the C-terminal half at amino acid position: 1,693–1,882 (E-value = 6.27e-20). In addition to these domains, other characteristic vitellogenin motifs were found: two polyserine regions, two putative RXXR consensus cleavage sites, and the GL/ICG and DGXR motifs near to the C-terminal end (Figure 2.1).

>BcVg1-like

MWSPIILGCLLVASTVLAGSHNNNNNNNRNNNNNRDNNKRSNNNESGNNARAWKQGQVYE
YQIQGRTLAALHDVADQYAGNLIKATLKVQAKNSDTVLAWVTNAKYSEVHANLSAGWKQE
IPDKHLNYQNWQISEKPFAIQYKNGVVRNLQVDKNTPTWELNIIKSIASQLQVDTRAENE
VSSRLNQKPKNGKNFGTFKTMEDTVTGETETLYDIKPLEQVEYQNKPQLAPMPNLKGSNG
ELIDIFKTKNFSRSETKTSYHFGIPGSNDVEPSSQVANVLARSSNSRIIIAGDLSHYTIQ
SSVTTDKIVISPELYNKQKGMVVSRMNITLNNVHSAAQNNAPALPSNVQKIEDLVYEFEP
ATSDESAQNNNRNNHHHHSDNKGDSSSSSSSGSSSSSSSSSSSSSSSSSSSESSSSS
SDSSNSSSGSSESAEKKNNRRHNNENNNKNSNENKKNQNNSNENSNENGKHNNSNESRNN
NGNNENSMSSERNNKKSRSRRPVIRNSNEESNENKRNQNNNSNSNENSNENQRQNNNNHN
DNNQHNHHHNDNKHNHNSGSSQSSSSSSSSSSSESGSSSSSSSSGSSSSGSSSEESHQPE
PKLNQAPQSPFLPFFIGNKGNNIQSSKQIEGVSAAKSLAQRIGQEIQDPNTISEHKTLSK
FTFLAGVVRTMNTKKMETATRELNYQQSKASKNSQSDAAKLNAWKAYRDAAAQAGTGPAL
IAIKQWIESGKVEGEEAAELLAVLPNTVRYPTREYIKEVFNLATSSQITKQGHLNTSAIL
SVASLARKAQVDSDNSHNQYPVHSFGKLSSKNSKDITERYTPYLANKLKEATRNQDSLKA
QVYIRALGNLGHPVILSVFKPYLEGKAPATNFQRLSMVAAMDQVARLFPKKVQSSLFNIY
LNQGESHELRCAAVFQLMKTNPSAAMLQRMAAFTEEDTSKQVNSAVKSAIESAAELKHPK
LQEFAQNAKAAKKMLSPKTQGLQYSKNFLSSYAMKEENLAYIKSINTIGSEDSLLPKGVF
VSLKRVLGGLEMEKDRFRAMTASVSDLLGLLENQFSTEESRKREKNAKQANQNDSHSTEK
IAQMLNIKREQAEQVEAQIFATILGGNRLFAFDNHTIEKIPQNLKAAAARLQNGQTFNYT
KLYNSYVINIGFPTATGLPFQFSYKKTHLVSAGGEMKLKTHPNIADANERQVVVPHSANV
SAEAQLLYASKSQSKISFITPYNQQRYIAGNNRFVQVNLPIKINADFDLQNGQVAFKVEP
LNKNQEQKLLIATQHPYTTIQNILNLSPAVKGQNTKDISVRETREWTETYGESSTGVAMK
VHYNGEDRAADLSTLYNKMQDSDLTTLAMFTNAREQINRNRFVVTYDGQRSSAKALRFYA
SYRNNEESRLRNADKNNQDNDNQNNNNNSPLEARASSAIPSSASRNGAEKRQEELLHKAS
AGIKDAQAAVLELMFEFEGNNNAQYVATAAVASSPADKNSKFLFYYQASPAQSSKFEMAV
AATAQVPQLSEMNFMKSEKEDPSVQINAEAMWGENAQSGAQIAIKAKLDQSEARKQYIAN
HPQAEQCRKQMEQRDNALPACRNMTARANALDEYTLNIKYEKVPQKLVNATYKLYQFARY
AGFAYNTENIVDVSNPSNQLKVRVNFADNHNSVNVSIEAPHGNAQFKNLPVPALAHHILI
QHPQYAIDERVGYYALNGQYTPVCVADGSSAQTFDNKTYPLNLEKDSWYVLMTSASKQRK
NNNVDYKTQRQNNVTILVKQSGDNKKDVQIILNNGEHVIEMQPASSSNNNNANAKIQVNK
KDQRASKNSVTEVTDSQNERIAQIYALPSEEVIVNIPSHGLSLNYDGSRVQVEANDRFRD
GVRGLCGTFNGEKATDFTTPRNCVVRDAKDFVASYSLSSNNRDNSRLSNNDFCAPQKQVR
FQQVINEKNAGSRSEAKRLNLQSIFGWMDFNNNNNESNSGNNSNNSNNNSNDSDNNNDNN
SNNHKNSRRQSKKNKNERNSEEYMNDESNNSAGKRNSQEQERNQQRGSTTHRLMVVESEN
QLCFSVKAIPQCNQGSRAQTPVEKKIDAHCVQDGQLPRQWREQARRGEPIAPIQRKTPNK
PMNVQVPTKCVAP*

Figure 2.1. Deduced amino acid sequence of *B. cockerelli* vitellogenin candidate (BcVg1-like) protein. Predicted features of the protein include, two polyserine regions highlighted in gray, two putative RXXR consensus cleavage sites shown in bold within red boxes, a motif GL/ICG bolded and underlined and two DGXR motifs in bold within blue boxes. This sequence was submitted to GenBank and its accession number is KX752432.

2.4.3 Phylogenetic analysis

A Bayesian analysis was conducted to evaluate the phylogenetic association of *BcVg1-like* with another animal Vgs (Figure 2.2). The analysis showed that *BcVg1-like* clustered within the insect vitellogenin clade and particularly it was placed in a clade formed by members of the hemipteran order. Also, the analysis showed that the clade formed by insect vitellogenins was more closely related to the arachnid and nematode clades than the clade formed by vertebrates and crustaceans Vgs. Interestingly, two aphid vitellogenins did not cluster within the insect clade.

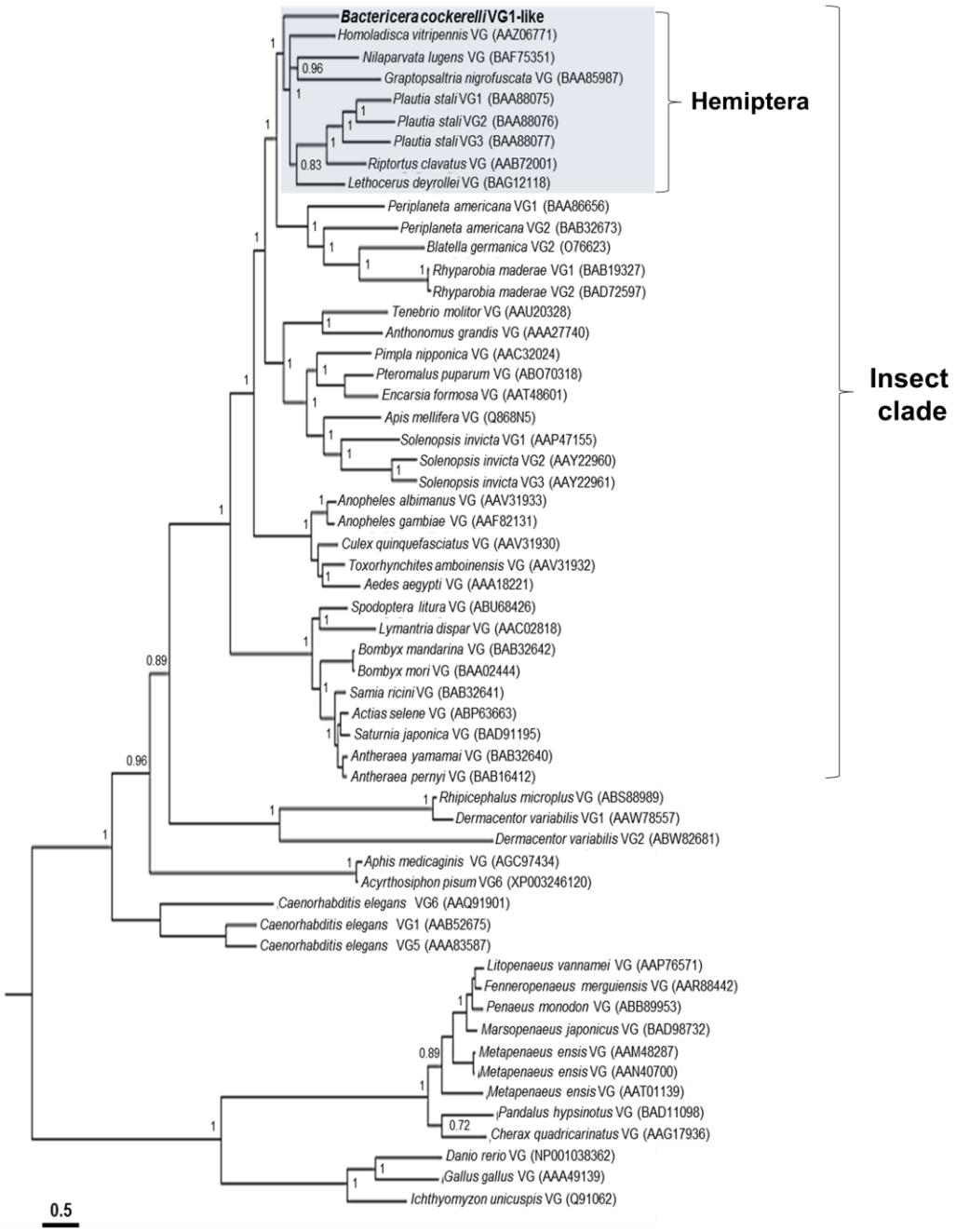

Figure 2.2. Phylogenetic analysis of *B. cockerelli* vitellogenin candidate and another animal vitellogenins. Bayesian analysis was conducted and the numbers at the nodes denote posterior probabilities, only values higher than 0.70 are shown. The reference bar indicates the distance (number of amino acid substitutions per site).

2.4.4 Expression of BcVg1-like in *Bactericera cockerelli*

Relative expression (fold change) analyses showed that BcVg1-like was expressed in adult females and its expression increased in virgin females up to 5 days. Expression in 5- and 7-day-old virgin females was 229 and 269 folds higher than in 1-day-old females, respectively ($P<0.001$), however there were no significant differences ($P>0.05$) between 5- and 7-day-old virgin females. Following mating, BcVg1-like expression was upregulated, and it was 445-fold and 1,339-fold higher in 5- and 7-day-old mated females relative to 1-day-old female respectively ($P<0.001$) (Figure 2.3A), the up-regulation of Vg expression was positively correlated with the amount of eggs oviposited by females (Figure 2.3C) (positive Pearson correlation coefficient of 0.98). The relative expression of BcVg1-like was also assessed in 4^{th}-5^{th} instar nymphs (pooled from both sex) and in 3- and 7-day-old males and females. While Vg expression was detectable in each life stage, the expression in males was significantly lower (10 fold) and the expression in 3- and 7-day-old females was higher (40 fold and 324-fold, respectively) relative to 4^{th}-5^{th} instar nymphs (Figure 2.4A).

Protein profiles of eggs, 4^{th}-5^{th} nymphs, 7-day-old males, 3-, 5- and 7-day-old virgin and mated females were compared. Two high molecular weight abundant bands corresponding to ~200 kDa proteins (highlighted with black arrows) were observed in 5- and 7-day old females but were absent in males (Figure 2.4B). Only one of these bands was observed in eggs and a faint band was present in nymphs. This expression profile and molecular weight were expected for Vg proteins following post-translational modifications. The identity of these bands as Vg was confirmed by mass spectrometry,

13 peptides matched the predicted protein sequence, representing a coverage of 11% of the total amino acid sequence (Figure 2.5). No differences were found between the LC/MS/MS profiles of the two bands.

Based on the comparison of protein profiles of virgin and mated females, the abundance of the ~200 kDa putative Vg proteins (highlighted with black arrows) was higher in 5- and 7-day-old virgin and mated females compared to 1-day and 3-day-old virgin females (Figure 2.3B). However, there were not apparent differences in abundance of Vg protein between virgin and mated females, which might be explained by differences in oviposition since mated females oviposited significantly more eggs compared to virgin females ($P<0.001$) (Figure 2.3C).

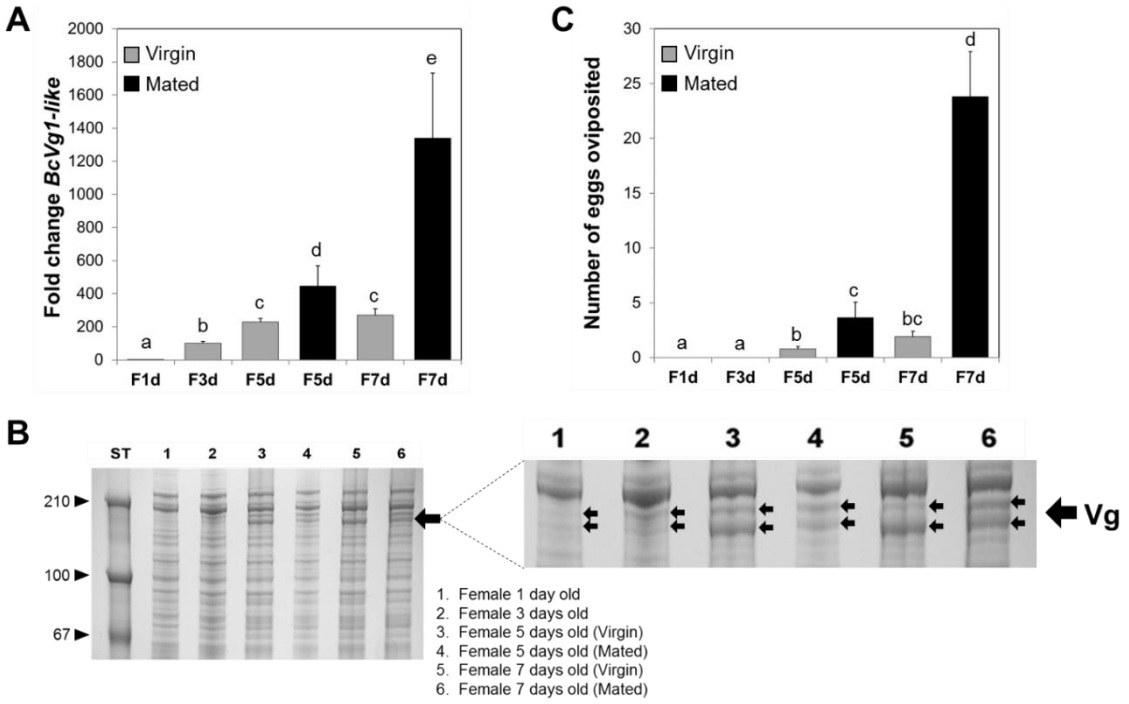

Figure 2.3. Expression analysis of *B. cockerelli* vitellogenin candidate in adult females. A. Relative expression levels of *B. cockerelli vitellogenin* transcript normalized to the expression value of *RPS18* and *Ef-1a* transcripts. Data represent means ± SD of three independent experiments. **B.** Protein abundance of *B. cockerelli* proteins (30 µg per lane) from females analyzed by SDS-PAGE (the experiment was conducted in triplicates). Two high molecular weight abundant bands corresponding to ~200 kDa putative Vg proteins are highlighted with black arrows. **C**. Amount of eggs oviposited on tomato plants by females. Data represent means ± SD of three independent experiments. Different letters indicate statistical differences between female adult-stages at $P<0.05$ using one-way ANOVA with Tukey's post hoc test. ST represents the protein molecular weight standard.

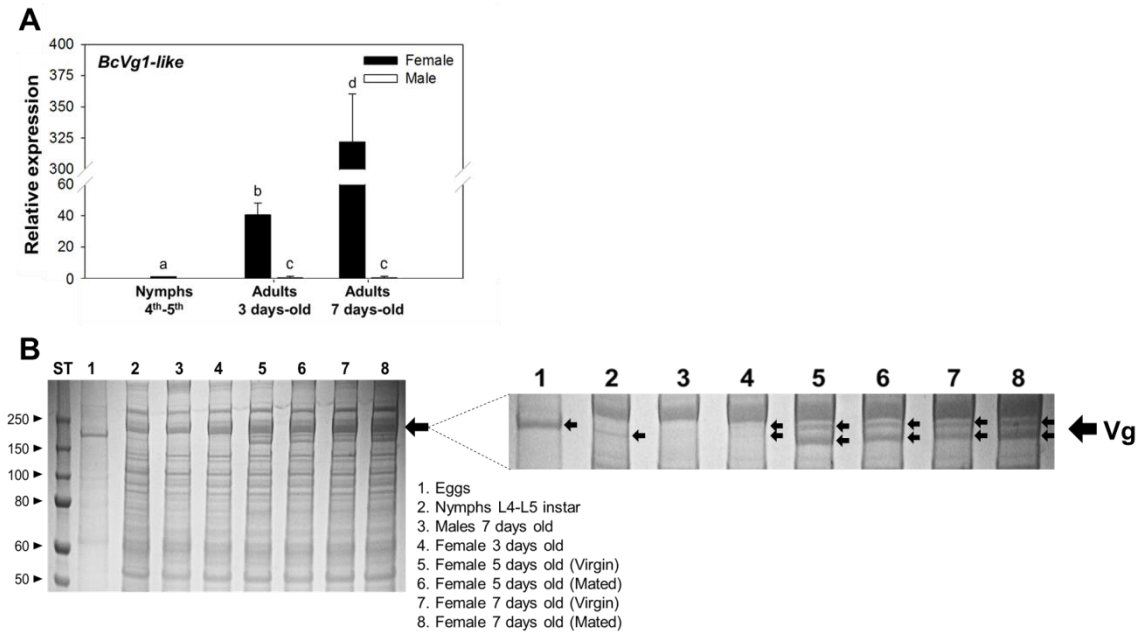

Figure 2.4. Expression analysis of *B. cockerelli* vitellogenin candidate in life stages. **A.** Relative expression levels of *B. cockerelli vitellogenin* transcript normalized to the expression value of *RPS18* and *Ef-1a* transcripts. Data represent means ± SD of three independent experiments. Data represent means ± SD of three independent experiments. Different letters indicate statistical differences between life stages at $P<0.05$ using one-way ANOVA with Tukey's post hoc test. **B.** Analysis of *B. cockerelli* proteins in different life stages (30 µg per lane) by SDS-PAGE. Two high molecular weight abundant bands proteins ~200 kDa corresponding to Vg were highlighted with black arrows. This experiment was repeated three times. ST represents the protein molecular weight standard.

>BcVg1-like

MWSPIILGCLLVASTVLAGSHNNNNNNNRNNNNNRDNNKRSNNNESGNNARAWKQGQVYE
YQIQGRTLAALHDVADQYAGNLIKATLKVQAKNSDTVLAWVTNAKYSEVHANLSAGWKQE
IPDKHLNYQNWQISEKPFAIQYKNGVVRNLQVDKNTPTWELNIIKSIASQLQVDTRAENE
VSSRLNQKPKNGKNFGTFKTMEDTVTGETETLYDIKPLEQVEYQNKPQLAPMPNLKGSNG
ELIDIFKTKNFSRSETKTSYHFGIPGSNDVEPSSQVANVLARSSNSRIIIAGDLSHYTIQ
SSVTTDKIVISPELYNKQKGMVVSRMNITLNNVHSAAQNNAPALPSNVQKIEDLVYEFEP
ATSDESAQNNNRNNHHHHSDNKGDSSSSSSSGSSSSSSSSSSSSSSSSSSSESSSSS
SDSSNSSSGSSESAEKKNNRRHNNENNNKNSNENKKNQNNSNENSNENGKHNNSNESRNN
NGNNENSMSSERNNKKSRSRRPVIRNSNEESNENKRNQNNNSNSNENSNENQRQNNNNHN
DNNQHNHHHNDNKHNHNSGSSQSSSSSSSSSSSESGSSSSSSSSGSSSSGSSSEESHQPE
PKLNQAPQSPFLPFFIGNKGNNIQSSKQIEGVSAAKSLAQRIGQEIQDPNTISEHKTLSK
FTFLAGVVRTMNTKKMETATRELNYQQSKASKNSQSDAAKLNAWKAYRDAAAQAGTGPAL
IAIKQWIESGKVEGEEAAELLAVLPNTVRYPTREYIKEVFNLATSSQITKQGHLNTSAIL
SVASLARKAQVDSDNSHNQYPVHSFGKLSSKNSKDITERYTPYLANKLKEATRNQDSLKA
QVYIRALGNLGHPVILSVFKPYLEGKAPATNFQRLSMVAAMDQVARLFPKKVQSSLFNIY
LNQGESHELRCAAVFQLMKTNPSAAMLQRMAAFTEEDTSKQVNSAVKSAIESAAELKHPK
LQEFAQNAKAAKKMLSPKTQGLQYSKNFLSSYAMKEENLAYIKSINTIGSEDSLLPKGVF
VSLKRVLGGLEMEKDRFRAMTASVSDLLGLLENQFSTEESRKREKNAKQANQNDSHSTEK
IAQMLNIKREQAEQVEAQIFATILGGNRLFAFDNHTIEKIPQNLKAAAARLQNGQTFNYT
KLYNSYVINIGFPTATGLPFQFSYKKTHLVSAGGEMKLKTHPNIADANERQVVVPHSANV
SAEAQLLYASKSQSKISFITPYNQQRYIAGNNRFVQVNLPIKINADFDLQNGQVAFKVEP
LNKNQEQKLLIATQHPYTTIQNILNLSPAVKGQNTKDISVRETREWTETYGESSTGVAMK
VHYNGEDRAADLSTLYNKMQDSDLTTLAMFTNAREQINRNRFVVTYDGQRSSAKALRFYA
SYRNNEESRLRNADKNNQDNDNQNNNNNSPLEARASSAIPSSASRNGAEKRQEELLHKAS
AGIKDAQAAVLELMFEFEGNNNAQYVATAAVASSPADKNSKFLFYYQASPAQSSKFEMAV
AATAQVPQLSEMNFMKSEKEDPSVQINAEAMWGENAQSGAQIAIKAKLDQSEARKQYIAN
HPQAEQCRKQMEQRDNALPACRNMTARANALDEYTLNIKYEKVPQKLVNATYKLYQFARY
AGFAYNTENIVDVSNPSNQLKVRVNFADNHNSVNVSIEAPHGNAQFKNLPVPALAHHILI
QHPQYAIDERVGYYALNGQYTPVCVADGSSAQTFDNKTYPLNLEKDSWYVLMTSASKQRK
NNNVDYKTQRQNNVTILVKQSGDNKKDVQIILNNGEHVIEMQPASSSNNNNANAKIQVNK
KDQRASKNSVTEVTDSQNERIAQIYALPSEEVIVNIPSHGLSLNYDGSRVQVEANDRFRD
GVRGLCGTFNGEKATDFTTPRNCVVRDAKDFVASYSLSSNNRDNSRLSNNDFCAPQKQVR
FQQVINEKNAGSRSEAKRLNLQSIFGWMDFNNNNNESNSGNNSNNSNNNSNDSDNNNDNN
SNNHKNSRRQSKKNKNERNSEEYMNDESNNSAGKRNSQEQERNQQRGSTTHRLMVVESEN
QLCFSVKAIPQCNQGSRAQTPVEKKIDAHCVQDGQLPRQWREQARRGEPIAPIQRKTPNK
PMNVQVPTKCVAP*

Figure 2.5. Protein identification analysis of a candidate Vg protein in *B. cockerelli*. Two high molecular weight abundant protein bands ~200 kDa that might represent Vg were digested and analyzed by LC/MS/MS, 13 peptides matched the predicted *in silico* digestion, they are underlined and highlighted in gray in BcVg1-like protein *in silico* prediction.

2.4.5 Effects of JH III applications

2.4.5.1 *Survival*

Three-day-old virgin *B. cockerelli* females were exposed to 6 JH III treatments. No significant effect on psyllid mortality was observed after 96 hours following treatment compared to the control (Log-rank P>0.05) (Figure 2.6).

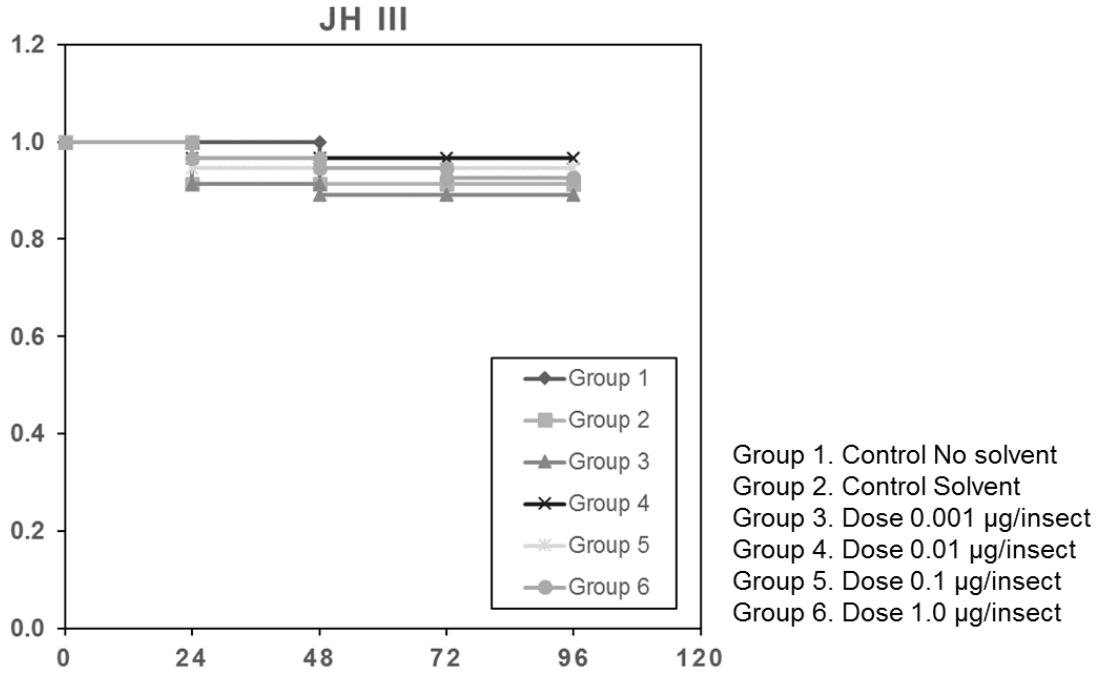

Figure 2.6. Survival of *B. cockerelli* females after exogenous application of JH III. Curves depict the Kaplan-Meier survival probabilities of psyllid females after applications of four JH III doses (0.001 µg, 0.01 µg, 0.1 µg and 1.0 µg of JH III/insect), and the respective controls (solvent and no solvent) during 96 hours. No difference in survival was observed (P>0.05).

2.4.5.2 *Number of mature oocytes*

The effect of JH III in *B. cockerelli* oocyte maturation was evaluated (Figures 2.7 and 2.8). A significant higher number of mature oocytes were observed in reproductive organs of virgin females exposed to any of the JH III doses 96 hours after the topical application (Figure 4) compared with controls (no solvent and solvent) ($P<0.001$).

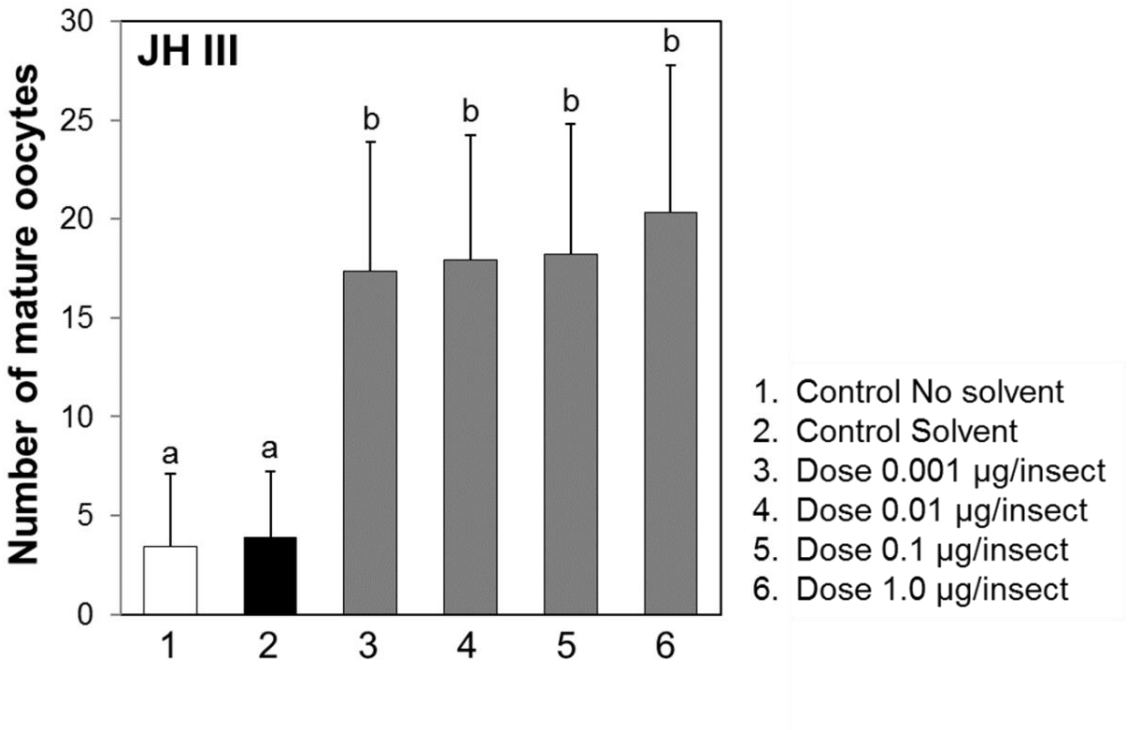

Figure 2.7. Average number of mature oocytes present in females 96 hours post-topical application of JH III. Treatments included four JH III doses 0.001 µg, 0.01 µg, 0.1 µg and 1.0 µg of JH III/insect and two controls, no solvent and solvent. Data represent means ± SD of three independent experiments. Different letters indicate statistical differences between treatments at $P<0.05$ using one-way ANOVA with Tukey's post hoc test.

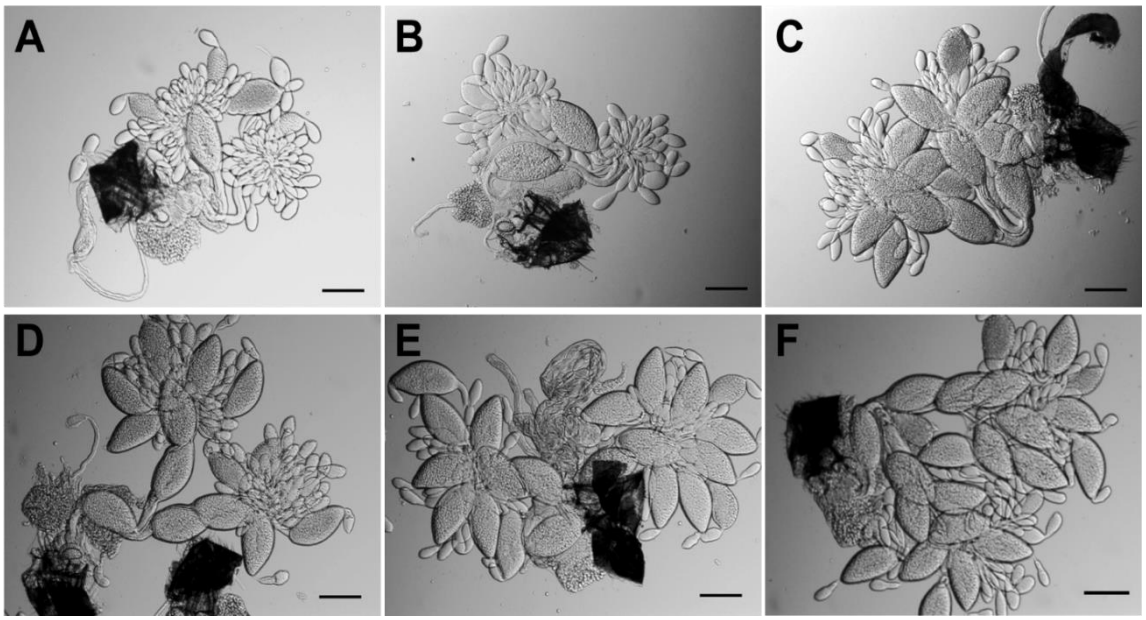

Figure 2.8. Representative image of ovaries from females treated with topical applications of JH III. Data represent the dissection of female reproductive organs 96 hours post-application of JH III. A and B are the no solvent and solvent controls, respectively, C to F correspond to females treated with 0.001 µg, 0.01 µg, 0.1 µg and 1.0 µg of JH III/insect, respectively. Scale bar is equal to 200 µm

2.4.5.3 *Oviposition*

A significant increase in the number of oviposited eggs in tomato leaves by virgin females treated with 0.01 µg of JH III/insect was observed after 96 hours compared with the 0.1 and 1.0 µg of JH III/insect treatments and the controls (No solvent and solvent) (P=0.003) (Figure 2.9).

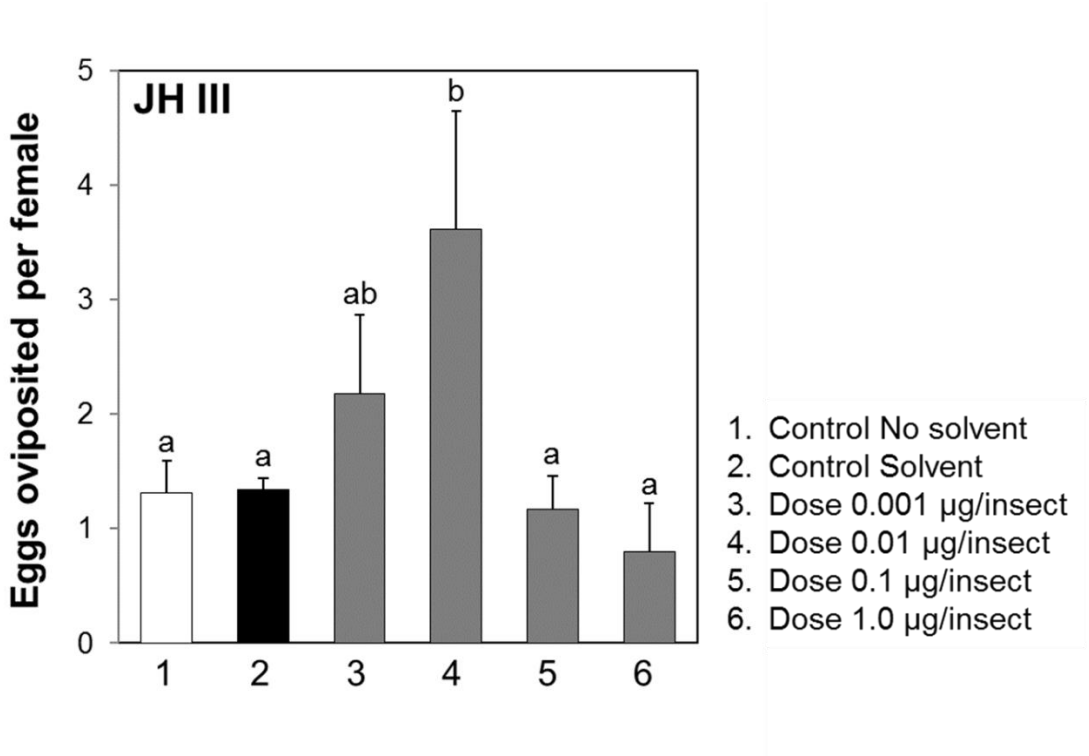

Figure 2.9. Number of eggs oviposited by females after topical application of JH III. Average of eggs oviposited per female during a period of 96 hours following treatment. Treatments included JH III application (0.001 µg, 0.01 µg, 0.1 µg and 1.0 µg of JH III/insect) and solvent and no solvent controls. Data represent means ± SD of three independent experiments. Different letters indicate statistical differences between treatments at $P<0.05$ using one-way ANOVA with Tukey's post hoc test.

2.4.5.4 *Vitellogenin transcript and protein expression*

In order to determine if juvenile hormone controls *BcVg1-like* expression in *B. cockerelli* females, the effect of treatments with 0.01 and 1.0 µg of JH III /insect on Vg expression was measured. These doses were chosen because 0.01 µg JH III/insect resulted in differences in oviposition while 1.0 µg/insect did not, compared to the controls. Fifteen hours after topical applications, there was a significant increase of the relative expression (fold change) of *BcVg1-like* transcript (Figure 2.10A) in samples treated with 0.01 µg and 1.0 µg of JH III/insect ($P<0.0001$). Overall, this result suggests that in *B. cockerelli* females, the transcription of vitellogenin might be under the control of juvenile hormone. For protein abundance analyses the four JH III doses were used. Protein abundance profiles of females showed an increase of the BcVg1-like protein in all samples treated with exogenous applications of JH III (Figure 2.10B, lanes 4 to 7) compared with controls 96 h after topical application (Figure 2.10B, lanes 2 and 3).

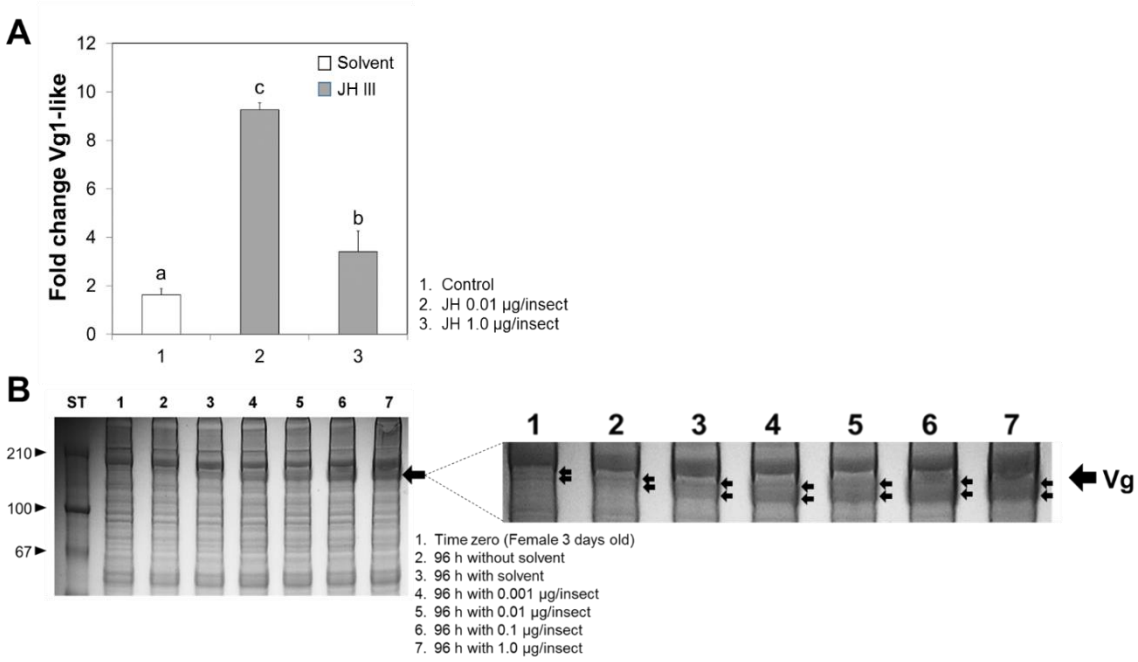

Figure 2.10. Expression analysis of *B. cockerelli* vitellogenin candidate after topical application of JH III in adult females. A. Relative expression levels of *B. cockerelli vitellogenin* transcript 15 hours post-application of JH III (0.01 µg and 1.0 of JH III/insect) or solvent, data was normalized to the expression values of *Rps18* and *Ef-1a* transcripts. Data represent means ± SD of three independent experiments. Different letters indicate statistical differences between treatments at $P<0.05$ using one-way ANOVA with Tukey's post hoc test. **B.** Protein abundance of *B. cockerelli* vitellogenin candidate after 96 hours post-application of JH III (0.001 µg, 0.01 µg, 0.1 µg and 1.0 µg of JH III/insect) and controls, protein samples (30 µg per lane) were analyzed by SDS-PAGE in triplicates, two high molecular weight abundant bands proteins ~200 kDa that might represent Vg were highlighted with black arrows.

2.5 Discussion

While vitellogenesis has been extensively studied in many insect species, very few studies have focused on phloem feeders (Tufail et al., 2010, Wang et al., 2010). In this study, one *B. cockerelli* Vg mRNA candidate was identified encoding a predicted 2,114 amino acid sequence long protein with several highly conserved Vg domains and motifs similarly to other insect Vgs (Tufail et al., 2014). Presently, in *N. lugens*, *Graptopsaltria nigrofuscata*, *L. deyrollei*, *Homalodisca coagulata* only one Vg transcript have been identified (Lee et al., 2000b, Tufail et al., 2010, Nagaba et al., 2011), while in other hemipterans such as *Riptortus clavatus*, *Plautia stali* and *Bemisia tabaci* more than one Vg transcript have been identified (Shinoda et al., 1996, Lee et al., 2000a, Wang et al., 2010). Based on the phylogenetic analysis, *B. cockerelli Vg1-like* clustered within the Vg hemipteran clade which in turn clustered with other insect orders (Figure 2.2). This bayesian analysis was similar to a neighbor-joining tree construction performed previously in (Tufail et al., 2014).

Analysis of *Vg* expression pattern in *B. cockerelli* revealed that *BcVg1-like* was highly expressed in females compared with males (Figure 2.4), similar expression pattern was described in *N. lugens* and *B. tabaci* (Tufail et al., 2010, Guo et al., 2012, Sun et al., 2016). Also, in *B. cockerelli* females, mating elicited an increase in the transcription level of *BcVg1-like* and this up-regulation was correlated with the amount of eggs oviposited by mated females (Figure 2.3A and 2.3C). Similar results have been reported in other arthropod species, including *L. maderae*, *Apis mellifera*, *Ornithodoros*

moubata and *Dermacentor variabilis* (Engelmann, 2002, Kocher et al., 2008, Horigane et al., 2010, Thompson et al., 2007).

In arthropods, Vg is transported via the hemolymph from the tissue of synthesis (commonly fat body) to the ovaries where it accumulates in the oocytes as vitellin (Raikhel, 1992, Friesen and Kaufman, 2004). Analysis of protein abundance in *B. cockerelli* females showed two bands ~200 kDa (Vg protein candidates) that increased in intensity from 1- to 7-day-old females (Figure 2.3B). However, changes in abundance of Vg protein between mated and virgin females were not apparent despite the increase in gene expression following mating. This discrepancy could be explained by the difference in number of eggs oviposited since mated females laid a higher number of eggs compared with virgin females (Figure 2.3C), and eggs contain high levels of vitellin.

Virgin females not only can produce mature oocytes, they also oviposited several eggs (Figure 2.3C). It is not well understood why *B. cockerelli* virgin females can produce and lay eggs. However, this was observed in other insects including *D. melanogaster* virgin females in which it is speculated that possessing mature oocytes might be useful to increase the body mass, and egg-laying is a default mechanism to produce fresh eggs under suitable conditions (Menon et al., 2014). Also, the presence of Vg protein in virgin females could have other explanations, aside from being involved on reproduction or egg production. For example in other organisms including insects, Vg protein might be related to other functions, such as transport of sugars, lipids, phosphates and hormones, as was described in previous studies cited in (Chen et al., 1997).

In insects, vitellogenesis is under a coordinate action of neuropeptides, juvenile hormone (JH) and/or ecdysteroids (Raikhel et al., 2002, Wyatt and Davey, 1996, Tufail et al., 2005, Nijhout, 2013), and hormonal differences have been observed in between mated and virgin females (Harshman et al., 1999, Bloch et al., 2000, Ogihara et al., 2007). In this study, we demonstrated that Vg expression in *B. cockerelli* females was up-regulated after topical application of JH III, suggesting that Vg expression is under the control of JH, as in other hemipterans such as *N. lugens*, *L. deyrollei* and *R. clavatus* (Shinoda et al., 1996, Tufail et al., 2010, Nagaba et al., 2011). However, in *R. prolixus* and *Oncopeltus fasciatus*, Vg can be expressed in the absence of JH (Pratt and Davey, 1972, Kelly and Hunt, 1982). Also, topical applications of JH III on *B. cockerelli* virgin females resulted in a significant accumulation of mature oocytes in the female reproductive tract. This enhanced mature oocyte accumulation has been reported in females of *D. virilis* (Raushenbach et al., 2004) and also in females of *B. tabaci* exposed to cotton plants treated with pyriproxyfen (JH analog) (Moshitzky and Morin, 2014). In these studies, two alternative mechanisms have been proposed to explain the accumulation of mature oocytes. The first mechanism is a 'physiological arrest of oviposition' (Raushenbach et al., 2004), and a the second mechanism involves muscle contraction in the oviduct, in which changes in the endogenous JH titers caused by pyriproxyfen, induced JH-dependent changes resulting in an imbalance in hormonal or neuro-hormonal regulation of myotropic factors, reducing the egg-laying process (Moshitzky and Morin, 2014). However, it is important to notice that there was not a mechanism that could explain why the egg-laying was modified by JH III in our system

and more analyses are required to fully understand this process. In particular, this effect was only observed in females treated with 0.01 µg of JH III/insect.

In conclusion, this study represents the first step to understand vitellogenesis in *B. cockerelli*. It highlights the role of JH III in the hormonal regulation of Vg production and oocyte development. Also, this information could have a critical relevance in the search for new strategies to control insect vectors.

3. *BACTERICERA COCKERELLI VITELLOGENIN-6 LIKE*, A VITELLOGENIN-LIKE PROTEIN WITHOUT A DIRECT REPRODUCTIVE FUNCTION?

3.1 Overview

Vitellogenins (Vgs), key proteins for insect reproduction, belong to a superfamily of large lipid transfer proteins (LLTP). During vitellogenesis these proteins provide carbohydrates, lipids, and other nutrients to the maturing oocytes. However, insects possess different number of Vgs gene copies (duplication events) suggesting a critical role of these genes in the insect species adaptation to its environment. In this study, we identified a new vitellogenin-like transcript in *Bactericera cockerelli, BcVg6-like*. *In silico* analyses predicted different conserved domains in *BcVg6-like* compared with the conventional *B. cockerelli* vitellogenin, *BcVg1-like*, previously described by our research group. Also, the expression analyses showed that *BcVg6-like* transcript was expressed at similar level between females and males and it was not up-regulated after exogenous application of juvenile hormone III (JH III). Our results suggest that in *B. cockerelli*, both Vg genes (*BcVg1-like* and *BcVg6-like*) may have different functions, one is a conventional Vg that conserved its ancestral function as an egg yolk-precursor and this new 'Vg-like' that might have acquired a new and still undetermined function.

3.2 Introduction

Potato psyllids, *Bactericera cockerelli* (Šulc.) (Hemiptera: Triozidae), damage solanaceous crops by feeding and/or by transmitting the plant pathogenic bacteria '*Candidatus* Liberibacter solanacearum', resulting in major losses in crops worldwide (Liefting et al., 2008, Teulon et al., 2009, Secor et al., 2009, Crosslin et al., 2010, Munyaneza et al., 2010, Munyaneza, 2012). In order to develop efficient control strategies that do not rely exclusively on pesticide applications, we are studying psyllid reproduction. Potato psyllids undergo sexual reproduction and females produce eggs, similar to other oviparous animals. One of the key processes for successful reproduction in oviparous species is the accumulation of yolk proteins into the oocytes during oogenesis. In most insects, yolk proteins (i.e. vitellogenins, Vgs) are synthetized in the fat body under the control of juvenile hormone. After their synthesis, Vg proteins are secreted into the hemolymph and they accumulate as vitellin in the oocytes via receptor-mediated endocytosis (Raikhel, 1992, Friesen and Kaufman, 2004). Vitellins are then used by the embryo during its development (Arukwe and Goksøyr, 2003).

Insect Vgs are multifunctional proteins that not only provide carbohydrates, lipids, and other nutritional supplies to the maturing oocytes (Chen et al., 1997). In addition, they have been associated with other functions not related to reproduction. For example, in *Apis mellifera,* vitellogenin has been associated with longevity, immunity and oxidative stress protection (Seehuus et al., 2006, Münch and Amdam, 2010, Havukainen et al., 2013).

Vitellogenins belong to a large lipid transfer proteins (LLTPs) superfamily. This diverse protein superfamily arose from a series of gene duplication events. Members of LTTP superfamily which include vitellogenins, apolipoproteins, and microsomal triglyceride transfer proteins, are involved in reproduction, lipid circulation, and immune defenses (Wu et al., 2013). Insect species harbor a variable number of Vg genes, ranging from one to several (Morandin et al., 2014). The difference in number of gene copies, which originated by duplication events, suggests a critical role in the insect species adaptation and might have important physiological or biological effects on gene function and regulation (Innan and Kondrashov, 2010). For instance, ancient gene duplication events in hymenopterans have resulted in three genes homologous to conventional Vgs that are classified as Vg-like-A, -B, and -C (Morandin et al., 2014).

Transcriptome and whole genome sequencing analyses of different insect species have revealed a new and diverse class of Vgs, the Vg-like genes. However, the study of these genes in hemipterans is still limited, in particular among phloem-feeding species such as *B. cockerelli, Nilaparvata lugens* and *Bemisia tabaci* (Ibanez et al., 2017, Tufail et al., 2010, Wang et al., 2010). While datamining a *B. cockerelli* transcriptome (Nachappa et al., 2012a), two putative Vg-like genes were identified, *BcVg1-like* (Ibanez et al. 2016) and *BcVg6-like.* In this study, we used *in silico* and molecular techniques to identify and characterize the *BcVg6-like* transcript, determine its expression pattern across psyllid life stages, and define its phylogenetic relationship to members of the large lipid transfer proteins superfamily.

3.3 Materials and methods

3.3.1 Insects rearing

Insect colonies from *B. cockerelli* northwestern haplotype were used and maintained as was previously described in (Ibanez et al., 2017). Briefly, adult psyllids of specific ages (3-day- and 7-day-old) were obtained by transferring 4th and 5th instar nymphs to 4-week-old tomato plants. When adults emerged (1-day-old), they were sexed under a stereo microscope (Olympus SZ-ST, Japan), and groups of ten 1-day-old females or males were relocated separately on different 4-week-old tomato plants for further analyses.

3.3.2 *BcVg6-like* transcript identification and cloning

The *Bactericera cockerelli* transcriptomic dataset (Nachappa et al., 2012a) was datamined to identify sequences (contigs) with similarities to vitellogenins using BlastX (Altschul et al., 1990). Two contigs with similarity to a predicted aphid vitellogenin-6 (Genbank accession XP_003246120) were identified.

For cloning, the full length *BcVg6-like* transcript was obtained in two steps. The first step was to identify the missing 3' end of the transcript, and it was achieved using a synthetized 3' Rapid Amplification of cDNA Ends (RACE) library from a pool of adults females and males (Takara Bio USA, Madison, WI) (Ibanez et al., 2017). RACE-PCR reactions were performed in a final volume of 50 µL, containing 50 ng of the 3' RACE cDNA, 5 µL of 10x Advantage 2 PCR buffer, 2 µL of 10 mM dNTPs, 2.5 µL (10 mM) of *BcVg6-like* RACE Fw1 primer (Table 3.1), 4 µL of Universal Primer A Mix (UPM;

Takara Bio USA, Madison, WI), 1 µL (1 Unit) of 50x Advantage 2 polymerase mix (Takara Bio USA, Madison, WI) and molecular grade water (Bioscience, VWR, Radnor, PA). The PCR conditions were 95°C for 1 min; followed by 35 cycles of 95°C for 30 sec, 68°C for 6 min and a final extension at 68°C for 6 min. The PCR amplicon of likely size was excised and purified from a 0.8% agarose gel using PureLink Quick Gel Extraction kit (Invitrogen, Carlsbad, CA). The PCR fragment (150 ng) was adenine-tailed and ligated into a pGEM-T easy vector using the pGEM-T Easy cloning kit (Promega, Madison, WI). The ligation was transformed into One Shot TOP10 competent cells (Invitrogen, Carlsbad, CA). Transformed colonies were selected and plasmid DNA extractions from at least three colonies were performed using PureLink Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad, CA). Then, these plasmid DNAs were sequenced by Eton Bioscience Inc. (San Diego, CA). The obtained sequences were compared to the *B. cockerelli* transcriptomic data to verify the sequence prediction. Then, in the second step, the complete ORF was amplified using primers designed upstream the start codon and downstream the stop codon (BcVg6FL Fw1 and BcVg6FL Rv2, respectively; Table 3.1). The PCR reaction was performed in a final volume of 50 µL, containing 50 ng of the 3' RACE cDNA, 10 µL of 5x Fidelity Buffer, 1.5 µL of 10 mM dNTPs, 1.5 µL of each primer (BcVg6FL Fw1 and BcVg6FL Rv2), 1 µL (1 Unit) KAPA HiFi™ polymerase (Kapa Biosystems, Boston, MA), and molecular grade water (Bioscience, VWR, Radnor, PA). The PCR conditions were 95°C for 3 min; followed by 35 cycles of 98°C for 30 sec, 60°C for 30 sec and 72°C for 2 min and a final extension at 72°C for 2 min. The PCR amplicon of the expected size (~4.3 kb) was excised, purified, cloned into a

pGEM-T easy vector, and sequenced as described above (GenBank accession number KY940305).

3.3.3 Bioinformatics and phylogenetic analyses

Full length *BcVg6-like* transcript was *in silico* translated using the Open Reading Frame Finder tool (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). Protein domains within the predicted Vg amino acid sequence were determined by NCBI Conserved Domain Database search (Marchler-Bauer et al., 2014).

Two alignments were performed to define the relationship of *BcVg6-like* with members of the superfamily of lipid transfer proteins using MEGA 5.2 software (Tamura et al., 2011). The first alignment was executed to determine the relationship between *BcVg6-like* with a previously described psyllid vitellogenin (*BcVg1-like*) (Ibanez et al., 2017) in addition to 56 animal Vgs within a large group of conventional Vgs.

For the second alignment and to identify orthologs of *BcVg6-like* a blast search was performed using the non-redundant protein database of the NCBI. This approach determined that *BcVg6-like* showed similarities to hemipterans and hymenopterans Vgs-like and uncharacterized proteins; these sequences were downloaded and aligned into a small database of LLTP superfamily sequences (n=66), represented by Crustacean apolipophorins, Crustacean vitellogenins and a subset of recognized insect conventional Vgs.

Then, to define the phylogenetic relationships of BcVg6-like, Bayesian inferences were assessed using each alignment on MrBayes 3.2 (Huelsenbeck and

Ronquist, 2001), following the parameters previously described (Ibanez et al., 2017). The runs were considered convergent when the average standard deviation was lower than 0.005 and the potential scale reduction factor value (PSRF) approached 1.0. The values of branch support were obtained by the method of posterior probability (Huelsenbeck and Ronquist, 2001). The trees were rooted at midpoint, and saved and edited using Figtree program v.1.4.0 (Rambaut, 2012).

3.3.4 RNA extraction and cDNA synthesis

Total RNA was isolated from pools of 10 insects (nymphs in 4^{th}-5^{th} instar, and 3-day- and 7-day-old females and males, respectively). For this, three independent replicates of insects were homogenized on ice for 1 minute using plastic pestles and 500 µL of TRIzol reagent (Life technologies, Carlsbad, CA) following the manufacturer's instructions. Genomic DNA contamination was removed from total RNA samples using Turbo DNase I (Life technologies, Carlsbad, CA) treatment following the company's instructions. The reverse transcription of complementary DNA (cDNA) was performed using 500 ng of total RNA, anchored-Oligo (dT) primers and Verso cDNA Synthesis kit (Thermo, Waltham, MA) following the manufacturer's instructions. After synthesis, cDNA samples were stored at -20º C until RT-qPCR analyses.

3.3.5 Gene expression analyses

The gene expression analyses were performed using RT-qPCR reactions with the SensiFAST SYBR Hi-ROX Kit (Bioline, Taunton, MA) following the company's

instructions, on an ABI 7300 real-time PCR Thermocycler (Applied Biosystems). For each RT-qPCR reaction; 5 ng of cDNA (template), 250 nM of each primer (Table 3.1) and 1x of SYBR Green Master Mix were used; the volume was adjusted with nuclease-free water to 10 µL. The real-time PCR program contained the following parameters: an activation step of 95°C for 2 min, then 40 cycles at 95°C for 5 sec and 60°C for 30 sec. Primers were designed using Primer3 (Untergasser et al., 2012). Each RT-qPCR reaction was performed in duplicates with their respective negative controls. The threshold cycle (Ct) values and efficiency of each primer set for RT-qPCR were determined using LinRegPCR software (Ramakers et al., 2003). Primer specificity was monitored with melting curve analysis using Sequence Detection System (SDS) software version 1.4.0.27 (Applied Biosystems) and 2% agarose gel electrophoresis. The *BcVg6-like* relative expression values were estimated using two reference genes; *ribosomal protein subunit 18* (GenBank accession KT279693) and *elongation factor-1a* (GenBank accession KT185020*)* as described (Ibanez and Tamborindeguy, 2016) and calculated using the delta delta CT method (Schmittgen and Livak, 2008).

Table 3.1. Primers used for *Bactericera cockerelli Vg6-like* identification and gene expression analyses.

Primers		
Purpose	**Name**	**Sequence**
RACE PCR	BcVg6-like RACE Fw1	5'-GGAGGACTTCTGCAACTTCTTCTCC-3'
Full length PCR	BcVg6FL Fw1	5'-TAACATGGACAACAAAGGATTGAA-3
	BcVg6FL Rv2	5'-CCTTTTTACATAAGGCCACATTTT-3'
Expression analysis	BcVg6-like qPCR Fw1	5'-ACGGAACTGTCTGGGAAATG-3'
	BcVg6-like qPCR Rv1	5'-TGGCGTTCAGTTCTTTGTTG-3'

3.3.6 Juvenile hormone III topical treatments

To determine whether the transcription of *BcVg6-like* is under the control of juvenile hormone (JH), 3-day-old virgin females were treated with JH III (Sigma-Aldrich, St. Louis, MO). Briefly, JH III was dissolved in a solution of acetone/ethanol (ratio 80:20) (Ibanez et al., 2017). Solutions (0.02 and 2.0 µg/µL) were freshly prepared from stocks for each replicate, then were topically applied at two different doses to the abdomen of ten 3-day-old virgin females (0.01 and 1.0 µg/insect) held in place during the procedure with the aid of a vacuum system. Then, the virgin females were transferred to 4-week-old tomato plants and 15 h post JH application they were flash frozen in liquid nitrogen and saved at -80°C until RNA extractions.

3.3.7 Statistical analyses

Statistical analyses of relative gene expression results were analyzed using one-way ANOVA with Tukey's post hoc test in JMP 12.0 software.

3.4 Results

3.4.1 Identification of *BcVg6-like* transcript

Two contigs that showed high similarity to aphid Vgs were identified in a *B. cockerelli de novo* transcriptome assembly (Nachappa et al., 2012a). These contigs possessed an overlap of 18 bases resulting in a 2,529 nucleotide unique sequence. This overlap was confirmed and validated by PCR. However, the ORF encoded in this sequence was truncated: it lacked a stop codon and it represented approximately the 5'-half of a predicted aphid Vg (Genbank accession XP_003246120). To obtain the complete open reading frame (ORF) of *BcVg6-like* a 3' RACE-PCR was performed. The primer was designed within the 5'-half. The obtained PCR fragment was cloned and sequenced, resulting in a 2,759 base-pair long amplicon. Based on the obtained sequence, primers were designed to amplify and validate the complete ORF. After PCR, we obtained a sequence that was annotated as *BcVg6-like* (GenBank accession number KY940305) encoding an ORF of 3,906 bases.

3.4.2 Bioinformatics analysis

In silico translation of *BcVg6-like* ORF, resulted in a putative protein of 1,301 amino acid residues (Figure 3.1). Domain architecture analysis of the BcVg6-like amino acid sequence by NCBI Conserved Domain Database (CDD) search (Marchler-Bauer et al., 2014) predicted a lipoprotein N-terminal domain at amino acid positions 27–583 (E-value = $2.03e^{-58}$). This is a large domain usually found in conventional Vgs. However, the CDD search neither identified a Domain of Unknown Function (DUF) motif 1943 nor a Von Willebrand factor type D domain found in conventional vitellogenins (Figure 3.2A).

A comparison of the motifs presents in a small group of Hemiptera Vgs-like highlights some of the differences between these proteins (Figure 3.3). BcVg6-like, *Acyrthosiphon pisum* Vg6-like and *Diaphorina citri* Vg-like amino acid sequences are similar in length and in the location of the lipoprotein N-terminal domain (LPD-N). While *Bemisia tabaci* Vg-like, and *Diuraphis noxia* Vg-like isoform X1 and X2 possessed in addition to the LPD-N, the von Willebrand type D domain (Figure 3.3).

>BcVg6-like

MDNKGLKLLLFASLALSLQEASSSQLFPNDRTYVFDWKVKFNTGAMLPSKM
VSEWHMNSKIIIQTSNFITQMQIVNVSGSHNINDTSLLNKPFRVNYTGGYI
SDLYIEPDDLPWSANMKRALASTFQVNLNLLKTRALVSSSREETIYGDCDI
DYVIQASAEDKLSLHKTVNLQSCVNLPINHWQNSPKYDCPSSFIDGSISLS
ERTMNFSLTDPVTIQNIVSSGIIEFQPFQAQAEVHHIYIKQYLTLENTDTQ
EKPAANLADVSNAIRTNILYEPLSDVDPIYGYKPSGDGILIEKVQTLLSEI
CDSLEWEIGIKGLDNETALQVLDLMWWLEKSEWEVLYNNITLGTSYSQETI
QHLFWDLLPQIGSNSSVIFIKDLVKTGRVKGFLAQRLVSTFPFYLRHPTEE
LLFQCEDLLRLGDNREIDAETRNASILSFATLIYKTCSVKCSVDVVDKYTK
IFLDRLTEASDYTHQMVYVHAISNMKLPKVLQFFAPQIANVSLSRHYRLLL
LWATMTAIDQSPELAKEIFWPILLNKTESLELRALSTLVLLTSSPTTARLS
AIHSVMIKEESQQLYNFYYTTLQSLAKTMMPCYAKINKVVSYFVRFVPPRT
HHWATGNYLLDYEDPVRGYGGLLQLLLLASEKTGLPNILHFTAEQHSLGVT
SSRSIYLKLEGINYKGLWDKFFGNGEVKVVKEPILREFIKQLNLHHLKQKQ
EHVHLEIFIKHDDHVIYCDYVNETVLDNIVTSIQKMSRVYLEFSVNYQSLQ
YPQRILTTQPTDIGTPALLTVTTSALLSARGSIQQKNDDDYVEKALPRTLR
NIELDVRYSINSITSLRTYNPLTNIWHGADRSRSLHARIPISTQLLLHYYR
SYYKITSKRHKYFKAGSRLGLVWHSTTHVVPNGLPTAENHKTEDDWSVESR
DLGARLGTLVFDCQDDARITDSLVIVKQAFQTHDKNYHMVLGGVPIVGLFS
LMNYFTFLPPGGSCGVVISLSPLEVEQKILVEGSTVSISMNQNNGTVWEMT
SSMRRLKDGDKEISLKFRHSNKELNAIDTNNTWRLIRLEGSLLIPSRKSGI
LKPPNAVRSQALISWGLSGNPSSMEFVIAPESKAQWPSDCSSNIPLCSQNT
SHFATKQKATLQYSNLPQWLKMAVRSVFSDFVKTEGNSTMFQFSFPSKLPW
TTRGMCAVNSKHLLTIDNTNVKTYDLKSECYSILLADCSVNSQFVLGIKKN
TNFKDLIDMKLLQGTTQLTNLTISANSSVIINGFLVELKDEYITYPINAKD
NDYIFKMKKWQETMIEIDMGNRGVIV*

Figure 3.1. Deduced amino acid sequence of the *B. cockerelli* vitellogenin candidate (BcVg6-like) protein. Predicted features of the protein include: LPD-N domain (highlighted in gray color). This sequence was submitted to GenBank and its accession number is KY940305.

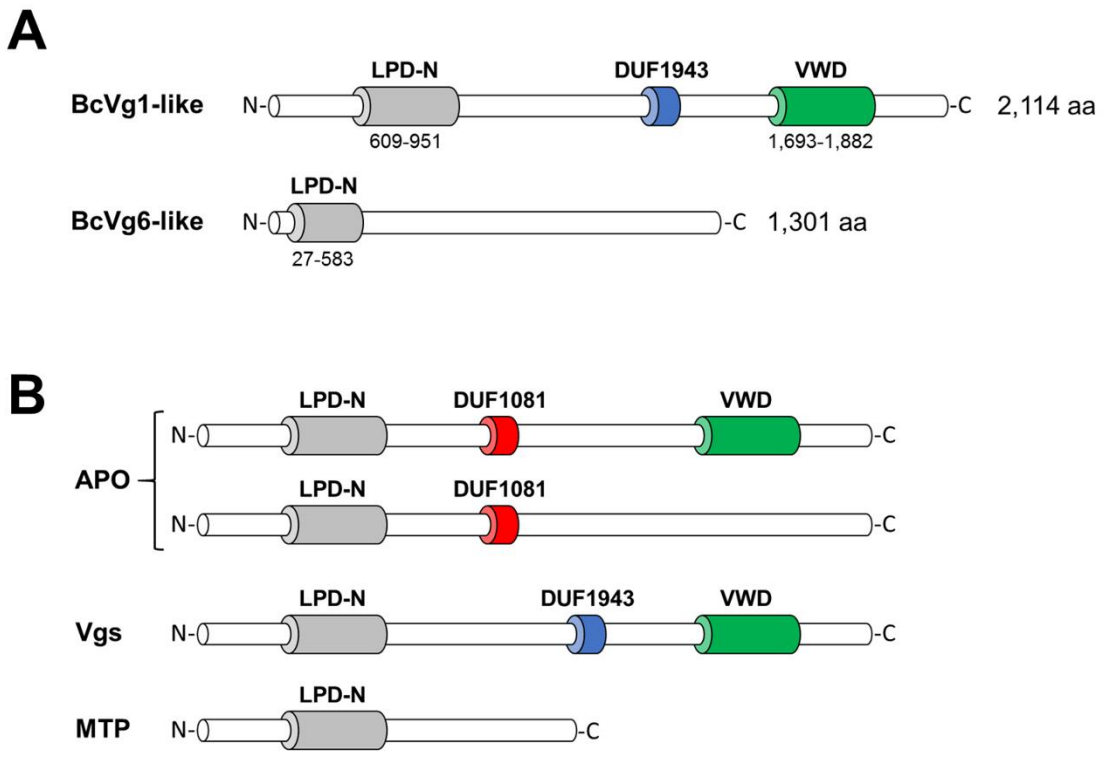

Figure 3.2. Structural domains present in members of the large lipid transfer protein (LLTP) superfamily. A. Comparison of structural domains found in *Bactericera cockerelli* proteins (*BcVg1-like* and *BcVg6-like*). These proteins possess the LPD-N domain, whereas only *BcVg1-like* has the predicted DUF1943 and VWD domains. **B.** Domains (LPD-N domain (light gray), DUF1081 (red), DUF1943 (blue) and VWD (green) domains) found within the large lipid transfer protein (LLTP) superfamily. The members of this superfamily include **APO** (including apolipoproteins, apolipocrustacein and apolipophorins), **Vgs** (vitellogenins) and **MTP** (microsomal triglyceride transfer protein).

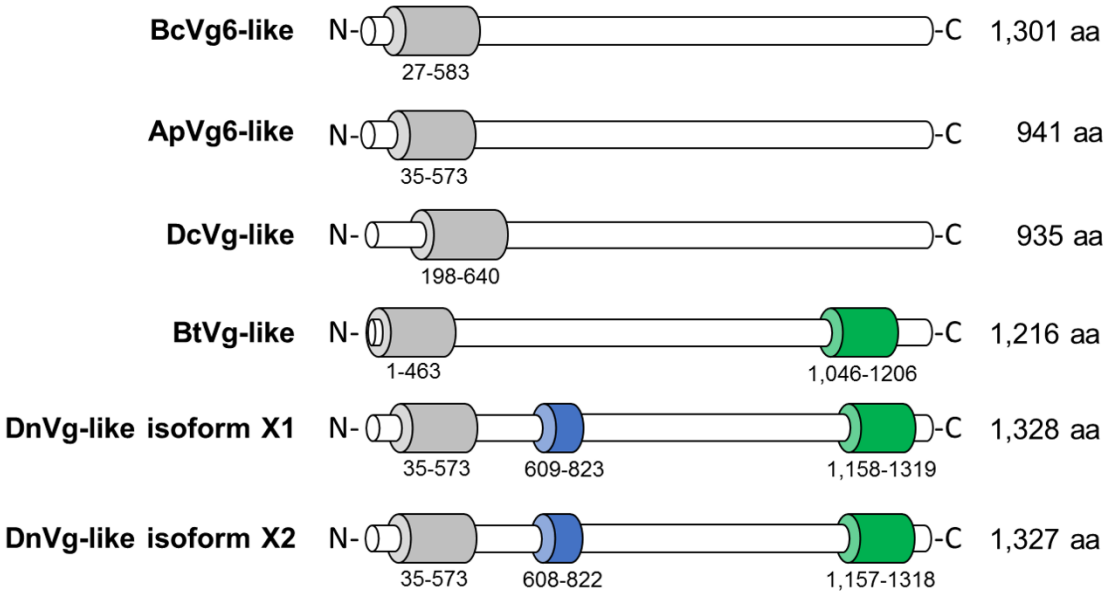

Figure 3.3. Graphical representation of motifs in the full length amino acid sequences of a small group of Hemiptera Vgs-like. Comparison of structural domains found in other phloem feeder hemipteran vitellogenins similar to *BcVg6-like*. Insect species included; **Bc**, *Bactericera cockerelli;* **Ap**, *Acyrthosiphon pisum;* **Dc**, *Diaphorina citri;* **Bt**, *Bemisia tabaci; and* **Dn**, *Diuraphis noxia*. The lipoprotein N-terminal domain is shown in light grey, the Domain of Unknown Function 1943 (DUF1943) is shown in blue, and the von Willebrand type D domain is shown in green.

3.4.3 Phylogenetic analysis

A phylogenetic study was conducted comparing *BcVg6-like* to members of the large lipid transfer proteins (LLTP) superfamily, using bayesian inferences. First a phylogenetic analysis focused in the relationship of *BcVg6-like* with conventional Vgs (Figure 3.4) was performed. In this analysis, *BcVg6-like* clustered within a clade formed by predicted aphid (Aphidoidea) Vgs, while *BcVg1-like* clustered within the conventional insect vitellogenins, specifically grouping with other hemipterans. The clade containing *BcVg6-like* did not cluster within the conventional insect vitellogenins.

The second phylogenetic analysis focused in the relationship of *BcVg6-like* with other members of a LLTP superfamily including the three new hymenopteran classes of Vgs (Vg-like-A, -B and -C). The results of this analysis identified two large clades. The first large clade included conventional insect Vgs, Crustacea Vgs and Crustacea apolipophorins. Whereas the second large clade was formed by the vitellogenin-like genes (Vgs-like A, Vgs-like B, Vgs-like C). *BcVg6-like* gene clustered with uncharacterized and annotated hemipteran vitellogenin-like genes forming a small group, that we called Hemiptera Vgs-like-B (Figure 3.5). This Hemiptera Vgs-like-group formed a sister clade with the described hymenopteran Vg-like B genes described in (Morandin et al., 2014). We propose to call this group Hemiptera Vg-like B group.

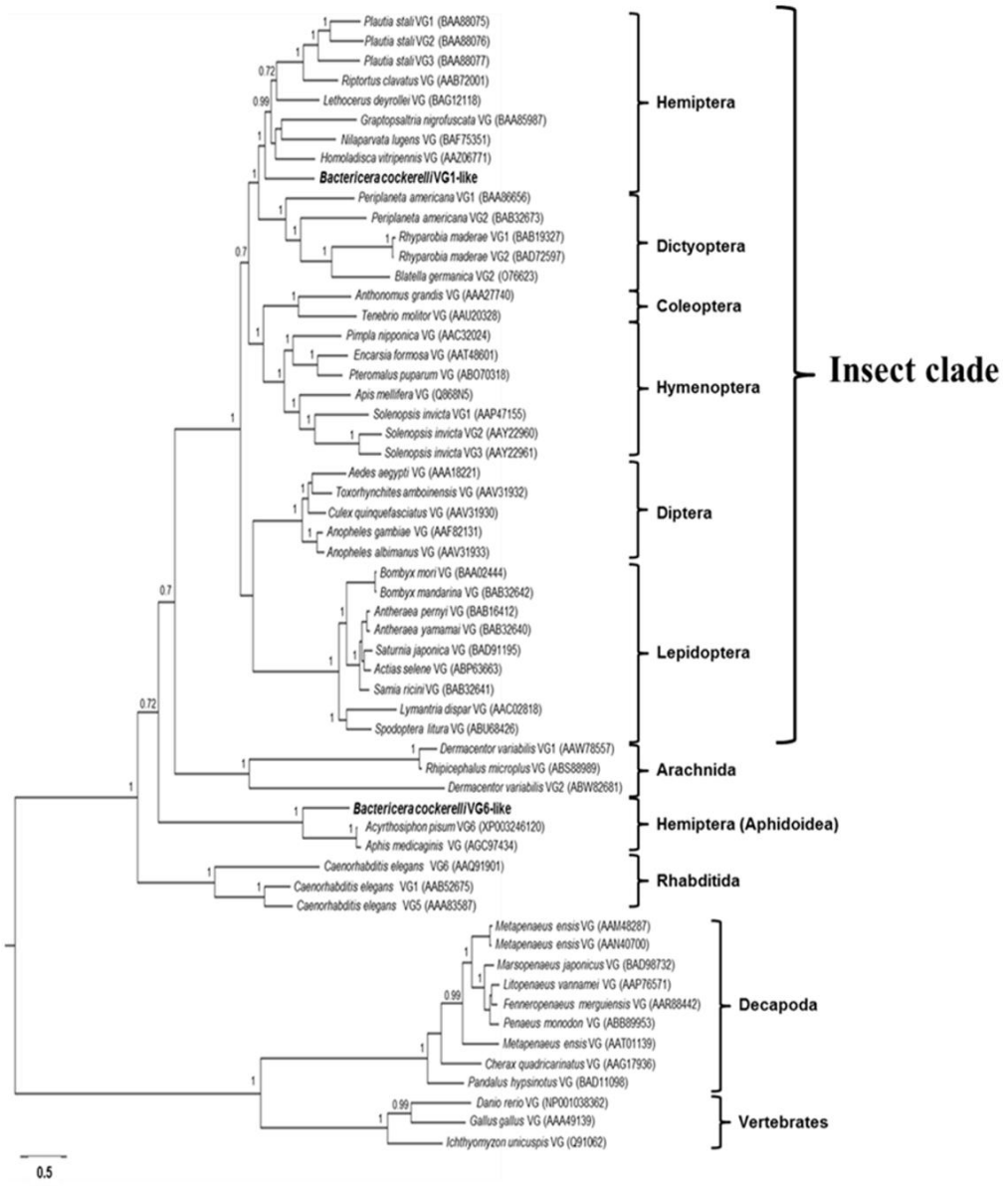

Figure 3.4. Phylogenetic analysis of *B. cockerelli* vitellogenin candidates (*BcVg1-like* and *BcVg6-like*). Phylogenetic analysis of *B. cockerelli* vitellogenins and other animal vitellogenins was performed using Bayesian inference. Accession numbers are given in parentheses and numbers at the nodes denote posterior probabilities (support values >0.7 are shown).

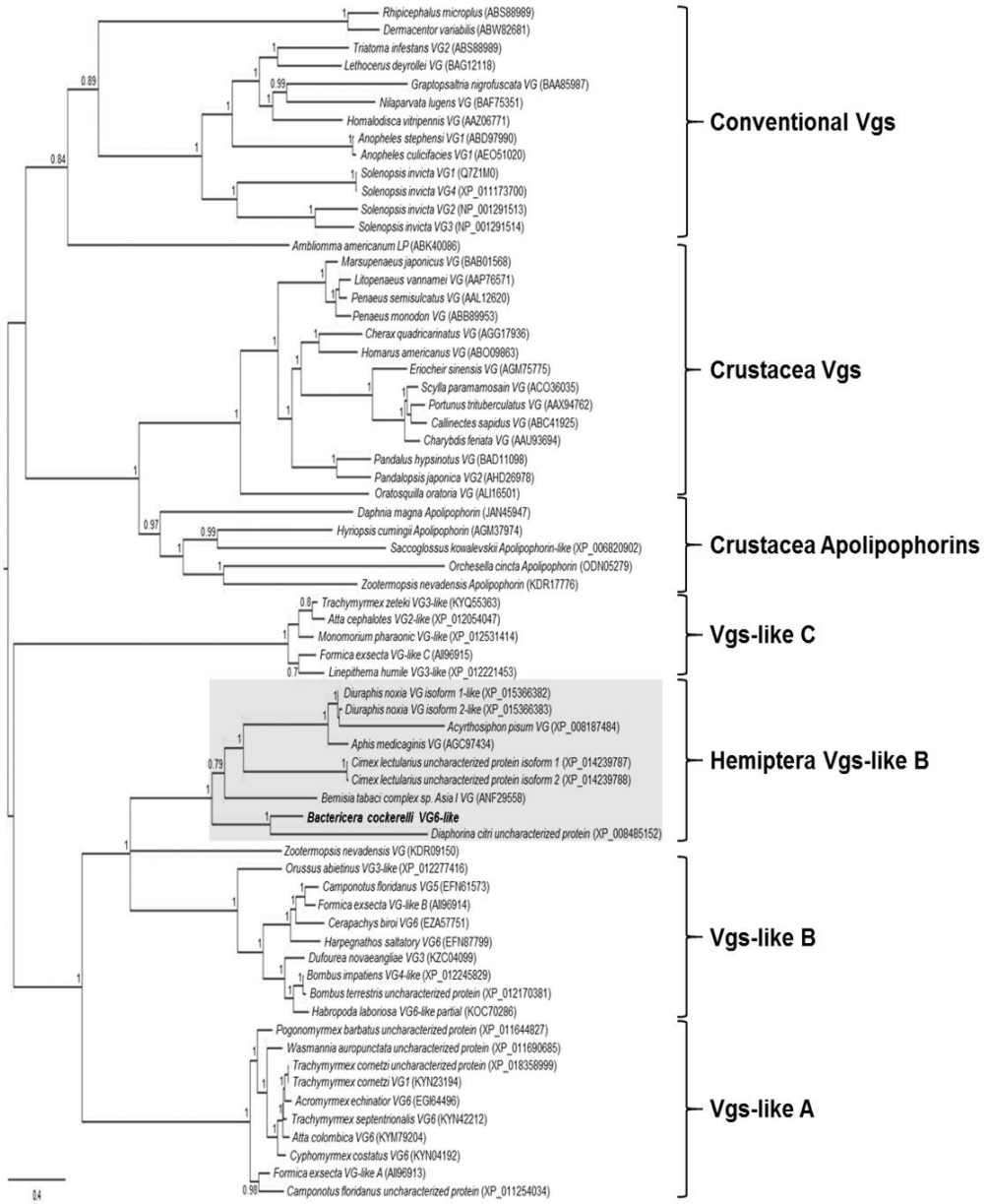

Figure 3.5. Phylogenetic relationship of *B. cockerelli vitellogenin 6-like* candidate with members of the large lipid transfer protein (LTTP) superfamily. Phylogenetic analysis of *BcVg6-like* and LLTP superfamily members was performed using Bayesian inference. Accession numbers are given in parentheses and numbers at the nodes denote posterior probabilities (support values >0.7 are shown).

3.4.4 Gene expression analyses

Relative expression analyses showed that *BcVg6-like* transcript was expressed in all life stages tested. Only significant differences in *BcVg6-like* relative expression were identified between 7-day-old males and 3- and 7-day-old females ($P<0.05$) (Figure 3.6A). Also, the pattern of relative expression of *BcVg6-like* was compared to the previously described *BcVg1-like* transcript expression (Ibanez et al., 2017) which was female-specific (Figure 3.6B).

To determine if the transcription of *BcVg6-like* was under the regulation of juvenile hormone, 3-day-old virgin females were treated independently with JH III at 0.01 or 1.0 µg/insect. Fifteen hours after the juvenoid application, *BcVg6-like* expression was analyzed (Figure 3.7A) and no differences in gene expression were identified between the controls and treatments ($P>0.05$). As a positive control, the relative expression of *BcVg1-like* transcript which is induced by JH III application (Ibanez et al., 2017) was also evaluated (Figure 3.7B).

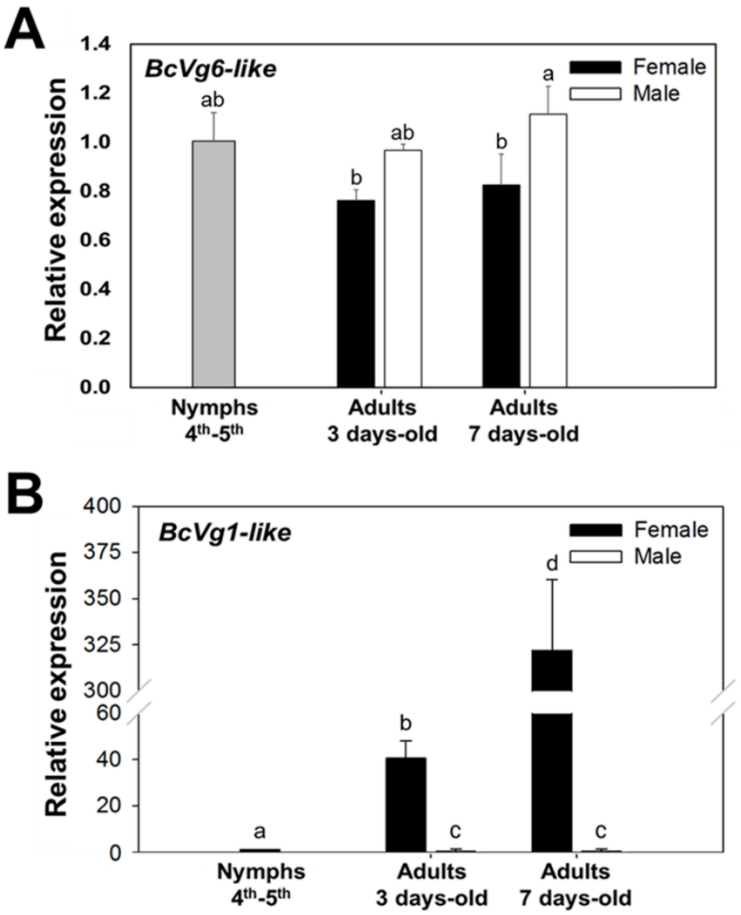

Figure 3.6. Expression analysis of *vitellogenin* candidates in different life stages. A. Relative expression levels of *BcVg6-like* transcript **B.** Relative expression levels of *BcVg1-like* transcript reproduced with permission from (Ibanez et al., 2017). Both relative expressions were normalized to the expression value of *RpS18* and *Ef-1a* transcripts. Each bar represents the means ± standard deviation (SD) of three independent experiments. Different letters indicate statistical differences between life stages at $P<0.05$ using one-way ANOVA with Tukey's post hoc test. While these analyses showed a significant induction in *BcVg1-like* transcript in females as they aged, these differences were not observed for *BcVg6-like* transcript.

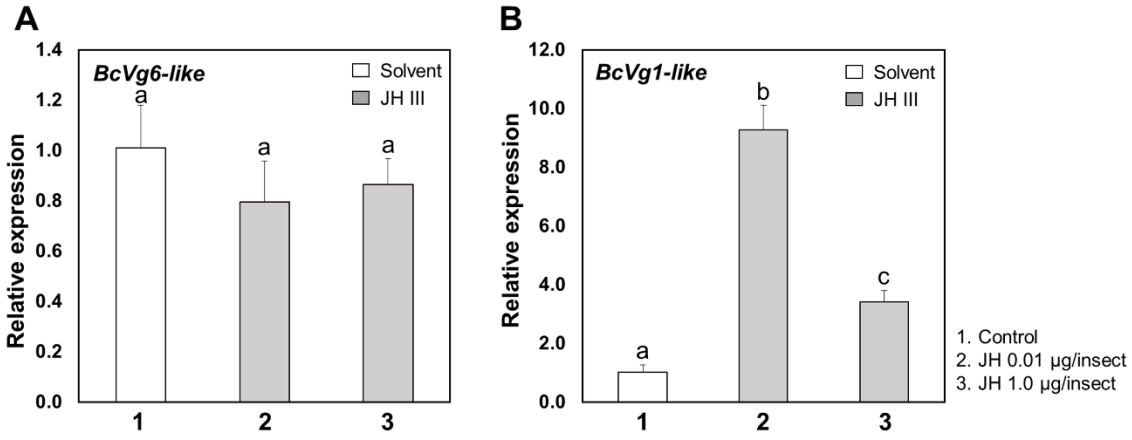

Figure 3.7. Expression analysis of *vitellogenin* candidates after topical application of JH III in adult virgin females. A. Relative expression levels of *BcVg6-like* transcript, showed no statistical differences with the control after JH application. **B.** Relative expression levels of *BcVg1-like* transcript reproduced with permission from (Ibanez et al., 2017), showed a significant upregulation in females post-JH application. Both transcripts were normalized to the expression values of *Rps18* and *Ef-1a* transcripts. Data represent means ± SD of three independent experiments. Different letters indicate statistical differences between treatments at $P<0.05$ using one-way ANOVA with Tukey's post hoc test.

3.5 Discussion

We previously reported that *B. cockerelli* possessed a vitellogenin transcript highly expressed in females after mating and after exogenous application of JH III (Ibanez et al., 2017). In this study, we identified a new putative *B. cockerelli* Vg-like transcript which was named *BcVg6-like* based in its similarity to a predicted vitellogenin-6 gene (Genbank accession XP_003246120) from *Acyrthosiphon pisum*. Analyses of the domain architecture showed that *BcVg6-like* has a large domain usually found within members of the LLTP superfamily, the lipoprotein N-terminal. However, *BcVg6-like* did not possess the motif DUF1943 nor did it possess the Von Willebrand factor type D domain commonly found in another insect Vgs, for instance *B. cockerelli* (*BcVg1-like*, Genbank accession KX752432), *Apis mellifera* (*AmVg*, Genbank accession NP_001011578) and *N. lugens* (*NlVg*, Genbank accession BAF75351) (Ibanez et al., 2017, Blank et al., 2013, Tufail et al., 2010). A study performed in zebrafish using recombinant protein assays, showed that DUF1943 domain interacted with lipoteichoic acid (LTA) of Gram-positive bacteria, as well with lipopolysaccharide (LPS) from Gram-negative bacteria, promoting the phagocytosis by macrophages (Sun et al., 2013). These results might suggest that conventional insect Vgs could also have a role in immune response.

In addition, other motifs found in most of insect vitellogenins such as cleavage sites and polyserine regions, were not identified in the *BcVg6-like* amino acid sequence. The polyserine regions (PSRs) in Vgs has been associated to high level of phosphorylation in these residues (Tufail et al., 2001, Tufail et al., 2005, Tufail and

Takeda, 2002). Also, the PSRs might be involved in solubility and cations (Ca^{2+} and Fe^{3+}) chelation (Gerber-Huber et al., 1987, Goulas et al., 1996). The phosphorylation of the PSRs seems to involved in binding to the vitellogenin receptor (VgR) as was showed in *Aedes aegypti*, and the dephosphorylation of these regions resulted in a binding impairment to VgR, indicating that the presence of phosphoserines might be required for adequate Vg uptake into the oocytes in maturation (Dhadialla et al., 1992). The differences in domains and motifs between *B. cockerelli* vitellogenins highlight the variability of the Vg sequence among oviparous organisms, and could explain the multiple physiological functions of Vg-like proteins that are not directly involved with egg production (Seehuus et al., 2006, Münch and Amdam, 2010, Havukainen et al., 2013). Because of the absence of the putative motifs involved in oocyte uptake and the fact that it is expressed in males we hypothesize that *BcVg6-like* might not be directly involved in egg production; its function might be involved with lipid circulation in *B. cockerelli*.

Based on the phylogenetic analyses (Figure 3.4) the *B. cockerelli* Vgs belong to different types of vitellogenins. Whereas, *BcVg1-like* clustered within the Vg hemipteran clade, within the insect conventional vitellogenins, *BcVg6-like* clustered with aphids Vg-like proteins clustering outside the insect Vg cluster. Further phylogenetic analyses (Figure 3.5) showed that *BcVg6-like* and other hemipteran Vgs clustered within Vgs-like B clade of Vgs-like proteins, that we called Hemiptera Vgs-like-B, forming a sister clade with hymenopteran Vg-like B proteins. These results support the hypothesis that an ancient gene duplication of a Vg precursor occurred in *B. cockerelli*.

In a recent study performed in *Formica* ants, three new classes of genes homologous to Vgs were reported, Vgs-like-A, -B, and –C (Morandin et al., 2014). *In silico* prediction analyses of these ant proteins showed that they differed in their conserved domains, and also in gene expression profiles. Previously, a study performed by our research group described one conventional potato psyllid Vg (*BcVg1-like*) gene that possessed a female-specific (sex-biased) expression. Moreover, the transcription of this gene was up-regulated by mating and exogenous application of juvenile hormone III (Ibanez et al., 2017). On the other hand, *BcVg6-like* was not female-specific (Figure 3.6) and its transcription was not up-regulated after exogenous applications of juvenile hormone III (Figure 3.7). The differences in conserved domains and completely different expression profiles between *BcVg1-like* and *BcVg6-like* suggest that *B. cockerelli* Vg orthologs may have evolved different functions after the duplication event, and these duplicated genes apparently had different fates, one a conventional Vg that conserved its ancestral function and this new 'Vg-like' that might have acquired new functions (neofunctionalization) (Prince and Pickett, 2002, Long et al., 2003). Until now, the biological functions of these Vg-like genes on insects remain unknown and these proteins might not be involved in vitellogenesis and/or egg production. The Vg-like proteins could have a role in lipid transport and/or other molecules. Interestingly, no conventional Vg has been identified in aphids so far. It is probable that in aphids, Vg-like proteins have retained the reproductive function. Studies of Vg knockdown and knockout, in addition to biochemical analyses, will be necessary to discover the functions of these Vg-like proteins in phloem-feeding hemipterans.

4. EFFECTS OF '*CANDIDATUS* LIBERIBACTER SOLANACEARUM' (HAPLOTYPES A AND B) ON *BACTERICERA COCKERELLI* (ŠULC) REPRODUCTION

4.1 Overview

'*Candidatus* Liberibacter solanacearum' (Lso) are phloem-restricted and unculturable gram-negative bacteria. Presently five haplotypes have been identified worldwide; but only haplotypes A and B are associated with potato psyllid, *Bactericera cockerelli* (Šulc.) in the Americas. Previous studies had showed that Lso-infection reduced the reproductive output in *B. cockerelli*. As a first step to understand the effect of Lso-infection in *B. cockerelli*, the expression of several genes involved in vitellogenesis were studied in LsoA- and LsoB-infected mated females. No statistical differences in the number of eggs oviposited and developing oocytes between Lso-free and LsoA-infected females were determined. In contrast, a significant reduction in the number of eggs laid and developing oocytes were determined in LsoB-infected mated females. Relative expression analyses showed that *BcVg1-like* and *BcKr-h1* were highly expressed in Lso-free females after mating (3-day-old females). A significant reduction of *BcVg1-like* and *BcKr-h1* was observed in LsoB mated females. A similar pattern of expression was detected for *S6 kinase*, *JH esterase* and *JH epoxide hydrolase* in Lso-free vs Lso-infected females across different ages. Overall, this study represents the first step to understand the interaction of Lso with *B. cockerelli*, highlighting the function of *BcVg1-like* and *BcKr-h1* in egg production and oocyte development.

4.2 Introduction

'*Candidatus* Liberibacter solanacearum' (Lso) are phloem-restricted and unculturable gram-negative bacteria transmitted by psyllids (Munyaneza et al., 2007a, Hansen et al., 2008, Liefting et al., 2008, Secor et al., 2009). Worldwide, five different Lso haplotypes have been described: LsoA, LsoB, LsoC, LsoD and LsoE (Nelson et al., 2011, Nelson et al., 2013, Teresani et al., 2014). Haplotypes A and B are associated with potato psyllid, *Bactericera cockerelli* (Šulc.) (Hemiptera: Triozidae) (Nelson et al., 2011) in the Americas and New Zealand, whereas the haplotypes C, D and E are associated with *Trioza apicalis* and *Bactericera trigonica* in the old world (Munyaneza et al., 2010, Teresani et al., 2014). '*Candidatus* Liberibacter solanacearum', have an important economic impact, resulting in major losses in crops worldwide (Liefting et al., 2008, Teulon et al., 2009, Secor et al., 2009, Crosslin et al., 2010, Munyaneza et al., 2010, Munyaneza, 2012). Despite the economic importance of Lso, their association with psyllids, their natural vectors, has not been studied in detail. In particular, critical physiological information is lacking about the effects of Lso infection on psyllid reproduction, specifically in egg production.

During oocyte development, yolk proteins (including vitellogenins), lipids, maternal RNAs, ribosomes, and organelles provide nutrients and/or patterning information for the future zygote (Chen et al., 1997, Arukwe and Goksøyr, 2003). In most insect species, juvenile hormone (JH) initiates the vitellogenic process and egg development (Tufail and Takeda, 2008), in particular in members of the Hemiptera order, such as *B. cockerelli*, *Nilaparvata lugens*, *Lethocerus deyrollei* and *Riptortus*

clavatus (Shinoda et al., 1996, Nagaba et al., 2010, Nagaba et al., 2011, Ibanez et al., 2017). Thus, a tight regulation of synthesis and degradation of JH are critical to achieve a precise JH titer. Three enzymes are involved in JH degradation in insects, JH esterase (JHE), JH epoxide hydrolase (JHEH), and JH diol kinase (JHDK) (Noriega et al., 2006, Cheng et al., 2014, Zeng et al., 2017), and these might play a role in the regulation of JH titers necessary for egg production. The synthesis of vitellogenins (Vgs), essential for egg production, occurs in most insects in the fat body. After synthesis, Vgs are secreted into the hemolymph and they accumulate in the developing oocytes by a receptor-mediated pathway, as vitellin proteins (Raikhel, 1992). In *B. cockerelli*, *BcVg1-like*, a vitellogenin gene with a reproductive function was described. BcVg1-like protein is highly expressed in females after mating and following exogenous application of juvenile hormone III (Ibanez et al., 2017).

The reproductive success of oviparous species depends on egg production. Insect are not an exception. However, egg production is energetically demanding and can be influenced by external or internal factors. Sufficient nutrients are need to initiate and complete egg production. The target of rapamycin (TOR) pathway has been proposed to integrate several signaling pathways and to be a central regulator of metabolism and physiology. TOR is a serine/threonine kinase that phosphorylate S6 protein kinase (S6K), which is one of the proteins responsible to transduce the nutritional signals and stimulate protein synthesis (including vitellogenin synthesis) in females (Hansen et al., 2005, Arsic and Guerin, 2008, Pérez-Hedo et al., 2013). Another physiological process that can directly affect the production of eggs in insects is immunity. Immune defenses

can have an antagonistic interplay with reproduction (Schwenke et al., 2016). Indeed, the activation of the immune system in insects after exposure to pathogenic bacteria resulted in a reduction of reproductive output (egg production) in many insect species such as *Drosophila melanogaster, Anopheles gambiae, Euoniticellus intermedius* and *Teleogryllus oceanicus* (Ahmed and Hurd, 2006, Reaney and Knell, 2010, Nystrand and Dowling, 2014, McNamara et al., 2014). Similarly, previous studies showed that Lso infection reduced reproductive output in *B. cockerelli*. Indeed, Lso-infected *B. cockerelli* females oviposited fewer eggs on tomato plants compared to uninfected females (Nachappa et al., 2012b, Nachappa et al., 2014, Yao et al., 2016). However, the molecular mechanisms that might affect the egg production in Lso-infected psyllids are still undetermined.

In this study, we examined the oviposition, number of developing oocytes, abundance of Vg protein and the transcriptional changes of genes related to vitellogenesis (*BcVg1-like*, *Krüppel homolog-1*), TOR pathway (*S6 kinase*) and JH degradation (*JH esterase* and *JH epoxide hydrolase*), in Lso-free, LsoA- and LsoB-infected virgin and mated females. Our objective was to start unraveling the cause(s) of the reduced reproductive output observed in Lso-infected *B. cockerelli* females.

4.3 Materials and methods

4.3.1 Plant Material

Tomato (*Solanum lycopersicum* cv. Moneymaker) were cultivated in a room (23 ± 3 °C) in plastic pots filled with Metro-Mix 900 (SUNGRO, Horticulture Distribution, Inc., Bellevue, WA, USA) and grown with a photoperiod of 16:8 h (Light: Dark). The plants were watered once every 2 days.

4.3.2 Insect colonies

The Lso-free *B. cockerelli* colony used for this study was obtained in 2013 from Dr. Don Henne, AgriLife Research Weslaco, TX. The colony was maintained on tomato plants in 14'' X 14'' X 24" insect cages (BioQuip, Rancho Dominguez, CA, USA) at room temperature and photoperiod of 16:8 h (Light: Dark). Insects from Lso-free colony were transferred to tomato plants infected with Lso A or Lso B in order to create laboratory psyllid colonies harboring Lso A and Lso B haplotypes. These colonies were tested regularly using SSR1 primers published by (Lin et al., 2012), previous to all analyses as was previously reported in (Yao et al., 2016) to verify the presence and the haplotype of Lso.

4.3.3 Insect adult cohorts

Cohorts of 1-, 3- and 7-days-old virgin females were obtained by transferring 4^{th}-5^{th} instar nymphs from the Lso-free, LsoA and LsoB colonies into different 4-week-old uninfected tomato plants. On the day adults emerged, they were sexed under a dissection

microscope (Olympus SZ-ST, Japan), then females and males of each colony were separated and kept as same age cohorts. To obtain 7-day-old mated females, groups of ten 1-day-old females from the Lso-free, LsoA or LsoB colonies were kept on 4-week-old uninfected tomato plants with ten 1-day-old males from the Lso-free colony, until they reached 7-days old. At day 7, the males were discarded and females were collected for follow-up analyses such as dissection of reproductive systems and VG gene and protein expression. Each 10-female group was considered a biological replicate.

4.3.4 Oviposition

Previously (second chapter) we determined that females and males are sexually mature and copulate when they reach 3-day-old. Therefore, groups of ten 1-day-old females and males, were caged together for seven days as was described above. On the seventh day, the number of eggs laid per group were counted. Also, on that day, the adults were collected, the females were used for further analyses (see below) and males were discarded. T. Eight biological replicates per treatment were performed.

4.3.5 Number of developing oocytes

The collected 7-day-old females from the oviposition study were anaesthetized on ice for 15 minutes, and their reproductive system was dissected using cold 1x Phosphate-buffered saline (1x PBS) in a dissection slide as previously described in (Ibanez et al., 2014). The dissected samples were transferred to a new 1.7 mL microcentrifuge tube previously filled with 200 µL of 3.8% formaldehyde/1x PBS buffer

(fixation buffer) for 1 hour. The fixation buffer was removed by washing the reproductive tissues twice for 15 minutes using 1x PBS. Then, the samples were mounted using 50 µL of Vectashield mounting medium with DAPI (Vector laboratories Inc., Burlingame, CA). The images of at least twenty reproductive systems per treatment were obtained using an Axioimager A1 microscope (Carl Zeiss microimaging, Thornwood, NY, USA) and visualized with AxioVision SE64 Rel. 4.9.1 software (Carl Zeiss).

4.3.6 RNA extraction and cDNA synthesis

Total RNA was extracted from pools of 10 insects per biological replicate (groups of ten 7-day old females) and homogenized during 1 minute on ice with a plastic pestle using 500 µL of TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Genomic DNA contamination was removed by DNase treatment using Turbo DNase (Ambion, Invitrogen, CA). Quantity and purity of the total RNA samples were determined using an Infinite® 200 PRO NanoQuant (Tecan, Männedorf, Switzerland) and RNA integrity was visualized by electrophoresis in agarose gels at 1.2% stained with ethidium bromide. Complementary DNA (cDNA) was synthesized using 300 ng of total RNA, anchored-Oligo (dT) primers and Verso cDNA Synthesis kit (Thermo, Waltham, MA) following the manufacturer's instructions. After synthesis, cDNA samples were stored at -20º C until RT-qPCR analyses.

4.3.7 Gene expression analyses by RT-qPCR

For gene expression analyses, RT-qPCR reactions were performed using PowerUp SYBR Green Master Mix according to manufacturer's instructions. Each reaction contained 5 ng of cDNA, 250 nM of each primer (Table 4.1) and 1x of SYBR Green Master Mix; the volume was adjusted with nuclease-free water to 10 µL. The real-time PCR program was 95°C for 2 min followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec. Primers were designed using Primer3 web (Untergasser et al., 2012). Real-time PCR assays were performed using an Applied Biosystems QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems). Three biological replicates per treatment were used and each RT-qPCR reaction was performed in duplicates with a negative control in each run. The threshold cycle (Ct) values and the primer specificity was monitored with melting curve analysis using QuantStudio™ software V1.3 (Applied Biosystems). The relative expression of the each transcript was estimated with the delta delta CT method (Schmittgen and Livak, 2008), using elongation factor-1a (GenBank *KT185020*) and ribosomal protein subunit 18 (GenBank *KT279693*) as reference genes (Ibanez and Tamborindeguy, 2016).

Table 4.1. Primers used for *Bactericera cockerelli* RT-qPCR relative gene expression analyses.

Primers		
Target gene	**Name**	**Sequence**
Vg1-like	BcVg1-like qPCR Fw1	5'-GACCTGTTGGGACTTTTGGA-3'
	BcVg1-like qPCR Rv1	5'-GCTTGTTTGGCGTTCTTCTC-3'
S6K	BcS6K-qPCR Fw1	5'-TTAGCTATGCCGCAGTTCCT-3'
	BcS6K-qPCR Rv1	5'-ACCGATTCTTGGGATTCCTC-3'
JHEH	BcJHEH qPCR Fw1	5'-ACATCCCCGACACCTACTG-3'
	BcJHEH qPCR Rv1	5'-CGACACATTGATGGTGAAGG-3'
JHE	BcJHE qPCR Fw1	5'-AGGTAGTTCCCGGCAACATG-3'
	BcJHE qPCR Rv1	5'-CTCCTTGTTGCCACCAAA-3'
Kr-h1	BcKr-h1 qPCR Fw1	5'-GAGTGGTCAGCTGGTGATCC-3'
	BcKr-h1 qPCR Rv1	5'-CACGTGAATCCCTTCCCACA-3'

4.3.8 Protein expression analyses

Protein samples were extracted from Lso-free, LsoA and LsoB females at the following specific ages 1-, 3- day-old (virgin) and 7-day-old (virgin and mated). Briefly, ten females were placed in a 1.7 mL microcentrifuge tube and homogenized on ice with a plastic pestle using 100 µL of 1x Radioimmunoprecipitation assay buffer (RIPA buffer) (Invitrogen, Carlsbad, CA) supplemented with protease inhibitor tablets (Mini, EDTA-free, EASY pack, Roche Diagnostics, IN, USA). Homogenized samples were incubated on ice for 30 minutes and centrifuged at 14,000g for 15 minutes at 4°C, then the supernatants were transferred to new tubes and the concentration of each sample was determined using Coomasie protein assay reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Twenty µg of each protein extract were loaded in gradient NuPAGE® Novex® 4-12% Bis-Tris (Invitrogen, Carlsbad, CA) gels and stained with SimplyBlue™ SafeStain (Invitrogen, Carlsbad, CA). Three biological replicates were performed per treatment (Lso-free, LsoA and LsoB) and the images of each gel were captured using a gel-documentation system (Fotodyne Incorporated, Hartland, WI). The intensity of the BcVg1-like protein bands was calculated using ImageJ software (Schneider et al., 2012).

4.3.9 Statistical analyses

The effect of Lso-infection in oviposition, number of developing oocytes, relative expression analyses and protein quantification were analyzed using one-way ANOVA with Tukey's post hoc test in JMP 12.0 software.

4.4 Results

4.4.1 Oviposition

The number of eggs oviposited on tomato leaves by groups of ten mated females from the Lso A, LsoB and Lso free colony were counted after seven days (Figure 4.1). No statistical differences were observed in the number of eggs oviposited between Lso-free and LsoA females ($P < 0.05$), both groups averaged 25 eggs laid per female. However, a significant reduction in the number of eggs oviposited by LsoB mated females was observed (average of 14 eggs per female) ($P < 0.05$).

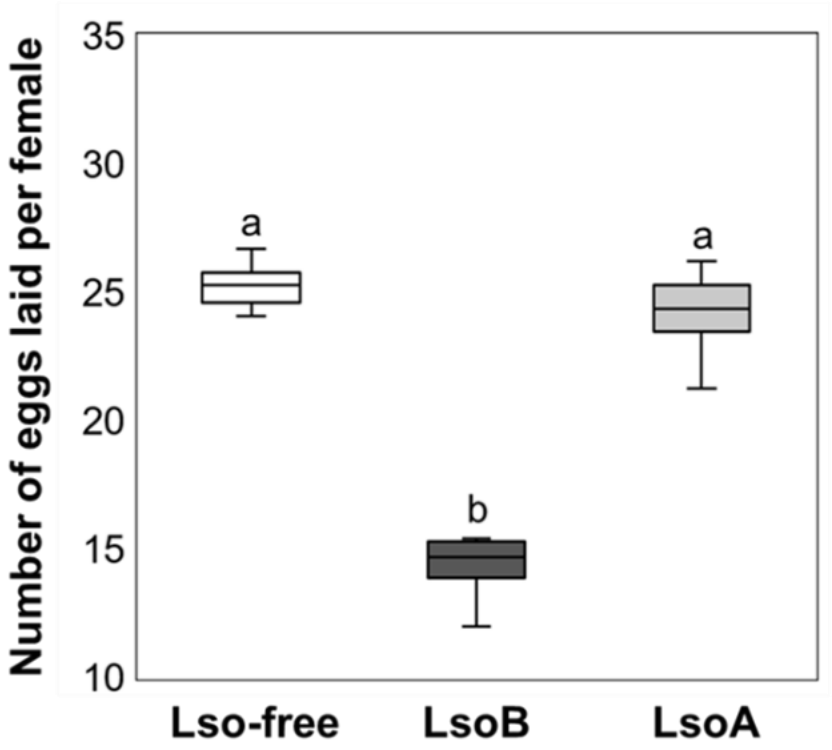

Figure 4.1. Average number of eggs oviposited by mated females from the Lso-free, LsoA and LsoB colonies on tomato leaves in a 7-day period. We observed no statistical differences in the number of eggs oviposited between Lso-free and LsoA-infected females. There was a significant reduction in the number of eggs laid by LsoB-infected mated females. Data represent means ± SD of eight independent experiments. Different letters indicate statistical differences between treatments using one-way ANOVA with Tukey's post hoc test ($P < 0.05$).

4.4.2 Developing mature oocytes

To evaluated a possible behavioral change in oviposition by LsoB females, such as egg retention, we counted the number of developing oocytes in 7-day-old Lso-free, LsoA and LsoB mated females (Figures 4.2 and 4.3). A significant reduction in number of developing oocytes were observed in the reproductive organs of the LsoB females (Figure 4.2), compared with Lso-free and LsoA females. No statistical differences were observed between the Lso-free and LsoA females.

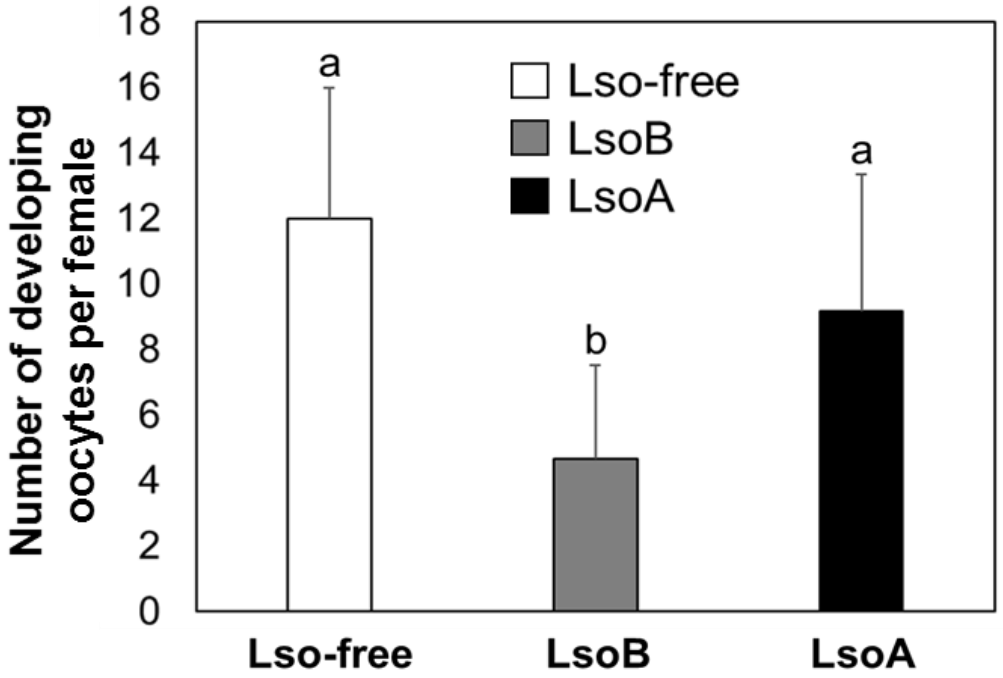

Figure 4.2. Average number of developing oocytes present in 7-day-old Lso-free, LsoA and LsoB mated females. We found a significant reduction in the number of developing oocytes determined in LsoB-infected mated females on dissected reproductive systems. Data represent means ± 20 reproductive female tissue samples. Different letters indicate statistical differences between treatments using one-way ANOVA with Tukey's post hoc test ($P < 0.05$).

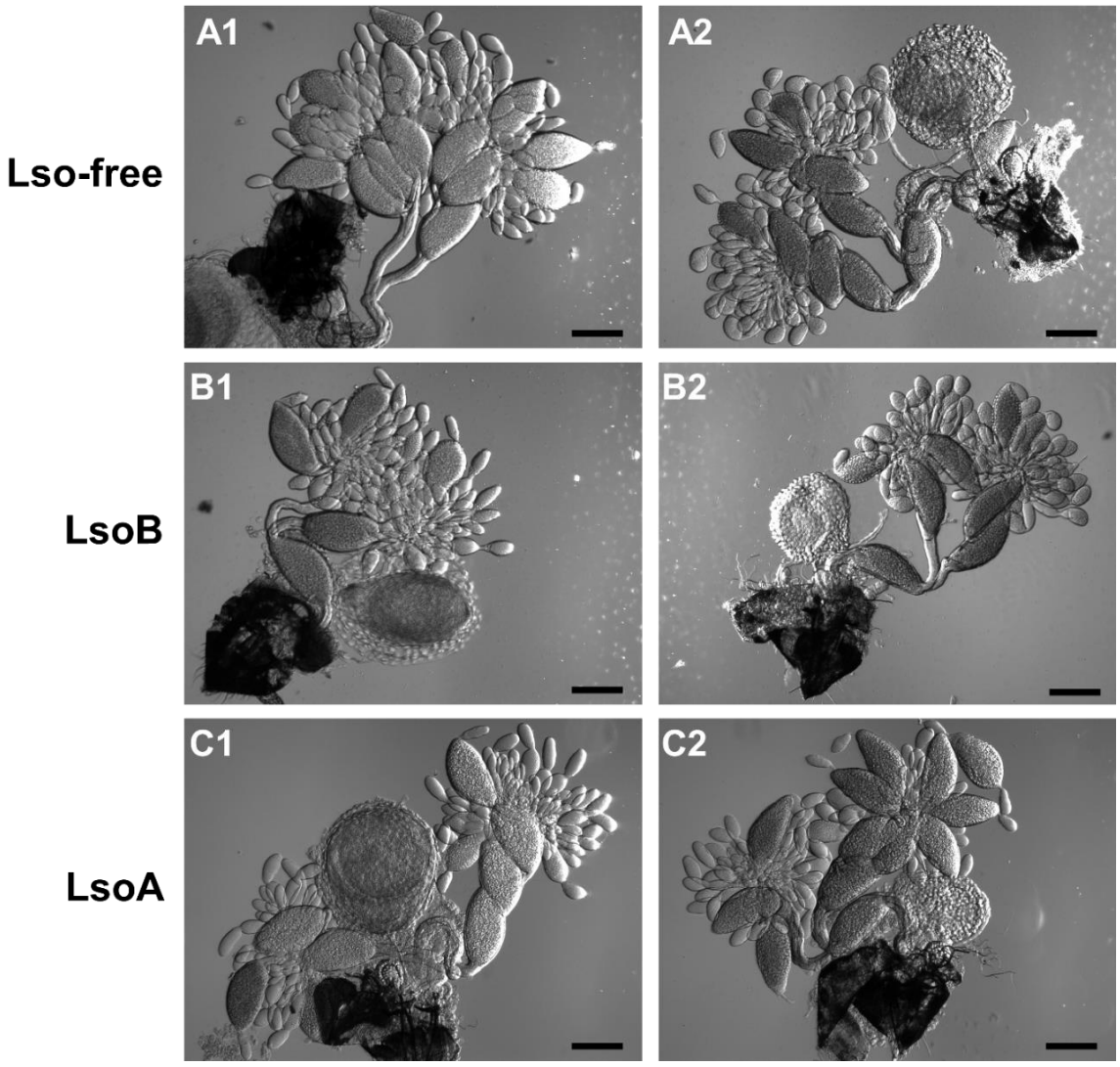

Figure 4.3. Representative image of female reproductive system from 7-day-old Lso-free, LsoA and LsoB mated females. The dissected ovaries showed reduction in the number of developing oocytes in LsoB-infected females. **A1** and **A2** are a representation of Lso-free mated females, **B1** and **B2** correspond to LsoB mated females and **C1** and **C2** are LsoA mated females. Scale bar is equal to 200 µm.

4.4.3 Analysis of gene expression in *Bactericera cockerelli*

Expression of transcripts *BcVg1-like* and *BcKr-h1* were compared among adult females of different ages from the Lso-free, LsoA and LsoB colonies. Relative expression analyses showed that *BcVg1-like* was expressed in all samples and its expression increased from 1-day-old virgin females to 7-day-old virgin and mated females (Figure 4.4A). *BcVg1-like* gene expression increased as females aged and following mated in Lso-free females. No differences in expression were found between 3- and 7-day old virgin females from the Lso A and Lso B colonies. However, a higher expression of *BcVg-1 like* was measured in 3-day-old LsoA virgin females than Lso-free virgin females, while in 7-day old virgin and mated females *BcVg1-like* expression was lower in LsoB females.

BcKr-h1 was also expressed in all samples (Figure 4.4B). In Lso-free females (white bars, Figure 4.4B) its expression significantly increased between 3-day and 7-day-old females. In LsoA females (black bars, Figure 4.4B), only in 7-day-old mated females *BcKr-h1* was up-regulated compared to younger or virgin LsoA females. In LsoB-infected females (gray bars, Figure 4.4B) this gene was not regulated, having similar level of expression in females of all tested ages (Figure 4.4B). No statistical differences in *BcKr-h1* expression were found when we compared 1- and 3-day old virgin Lso-free vs LsoA- and LsoB-infected females. However, a significant reduction in *BcKr-h1* gene expression was observed in 7-day-old virgin females LsoA- and LsoB-infected females compared to Lso-free females. While, in 7-day-old mated females the down-regulation was only observed in LsoB-infected females. Overall LsoB infected

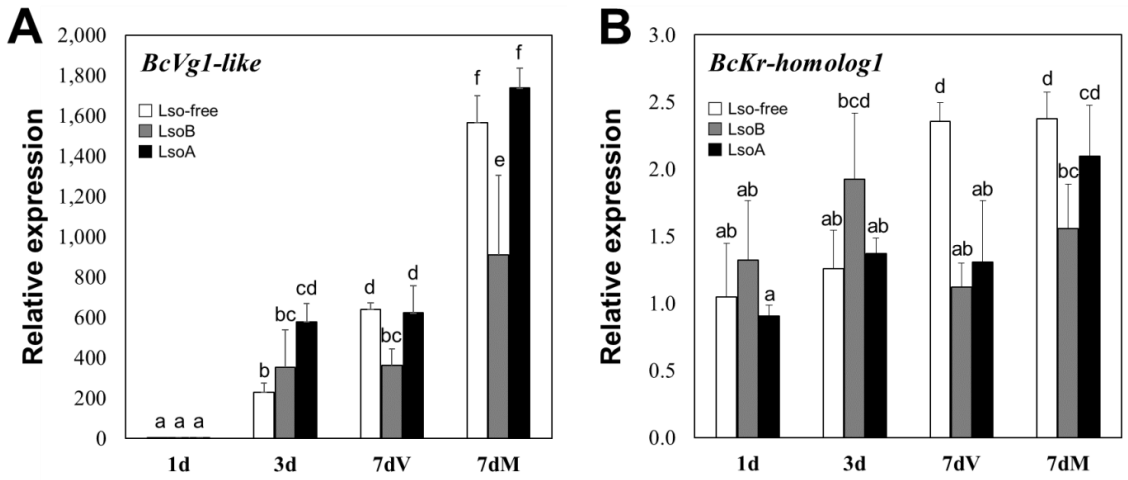

Figure 4.4. Vitellogenesis-related transcripts expression analyses in Lso-free, LsoA and LsoB *B. cockerelli* females of different ages. Relative *Vitellogenin 1* and *Krüppel homolog-1* transcript expression was determined by RT-qPCR and normalized to the expression value of *RpS18* and *Ef-1a* transcripts. **A**. *Vitellogenin 1* (*BcVg1-like*) had a reduced expression in LsoB-infected females. **B**. *Krüppel-homolog1* (*BcKr-homolog1*) had a reduced expression in LsoB-infected females. Each bar represents the means ± standard deviation (SD) of three independent experiments. Different letters indicate statistical differences between life stages using one-way ANOVA with Tukey's post hoc test ($P < 0.05$).

females had a reduction in *BcVg1-like* and *BcKr-h1* expression compared to Lso-free and LsoA females.

The transcriptional change of S6K, a gene involved in ribosome recruitment to 5′-capped mRNA, and one of the Target of rapamycin (TOR) substrates, was analyzed in Lso-free, LsoA and LsoB *B. cockerelli* females of different ages (Figure 4.5). Significant differences in the expression of this gene was found among females of different ages. *S6 kinase* expression increased in 3-day old females and decreased in 7-day-old virgin females compared to 1-day old females. Higher expression was found in 7-day-old mated females compared to 7-dayl old virgin females. Differences in gene expression among the different Lso-infected females were observed in 3-day-old virgin females.

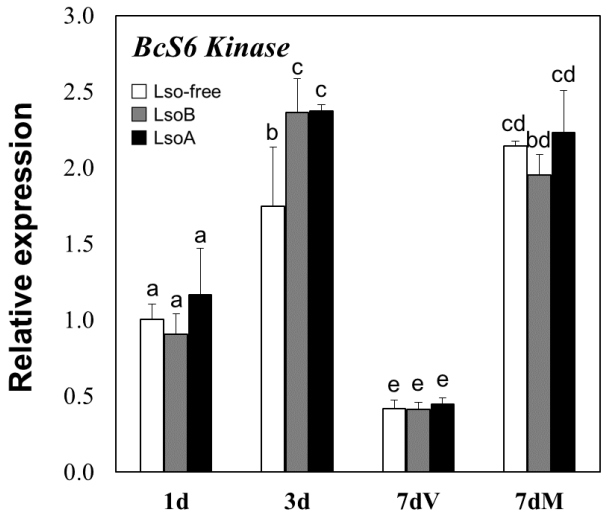

Figure 4.5. *BcS6K* mRNA expression analyses in Lso-free, LsoA and LsoB *B. cockerelli* females of different ages. Relative S6K mRNA levels were determined by RT-qPCR and normalized to the expression value of *RpS18* and *Ef-1a* transcripts. We observed similar pattern of expression in Lso-free vs Lso-infected females across samples, except in 3-day-old females. Each bar represents the means ± standard deviation (SD) of three independent experiments. Different letters indicate statistical differences between life stages using one-way ANOVA with Tukey's post hoc test ($P < 0.05$).

Juvenile hormone III was shown to be involved in *B cockerelli* reproduction, inducing the level of vitellogenin expression (Ibanez et al., 2017). This insect hormone is critical in several aspects of the insect physiology and its titer, which plays an important role in reproduction (vitellogenesis and oogenesis), depends on its biosynthesis and its degradation. In this study, we examined the relative expression of two genes involved in JH degradation; *juvenile hormone esterase* (JHE) and *juvenile hormone epoxide hydrolase* (JHEH) (Figure 4.6). A significant reduction in the level of expression of *JHE* transcript from 1-day-old females compared to the other ages was measured. However, no changes of *JHE* transcript expression were observed when comparing among same age females, except between 1-day old LsoA and LsoB females (Figure 4.6A).

The transcript expression of *JH epoxide hydrolase* (JHEH) showed higher expression in 7-day old virgin Lso-free females compared to 1-day-old and 7-day-old mated females; and in 7day-old virgin LsoB females compared to 1-day-old and 3-day old females. Only differences between 7-day-old LsoA and LsoB virgin females were observed (Figure 4.6B). Overall, the expression of genes involved in JH degradation were not affected by the presence of Lso in *B. cockerelli*.

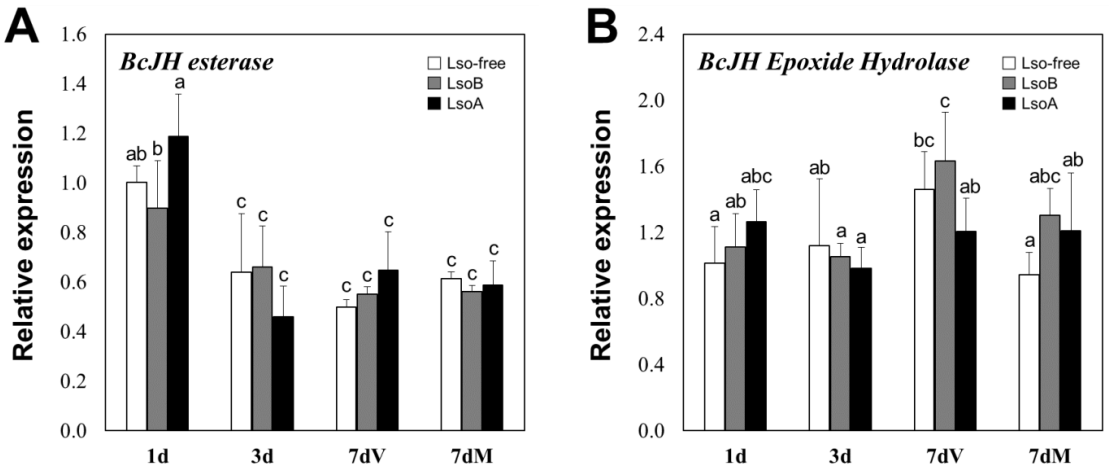

Figure 4.6. Juvenile hormone metabolism mRNAs expression analyses in Lso-free, LsoA and LsoB *B. cockerelli* females of different ages. It was observed for *JH esterase* and *JH epoxide hydrolase* similar pattern of expression in Lso-free and Lso-infected females across samples. Relative *JH esterase* and *JH epoxide hydrolase* mRNA levels were determined by RT-qPCR and normalized to the expression value of *RpS18* and *Ef-1a* transcripts. Each bar represents the means ± standard deviation (SD) of three independent experiments. Different letters indicate statistical differences between life stages using one-way ANOVA with Tukey's post hoc test ($P < 0.05$).

4.4.4 Protein expression analyses

To understand whether or not Lso infection modifies the abundance of BcVg1-like protein in *B. cockerelli* females, we investigated its expression pattern in 1-day, 3-day, and 7-day-old virgin and mated females from the Lso-free, LsoA and LsoB colonies (Figure 4.7). The abundance of the ~200 kDa putative Vg proteins (highlighted with black arrows) was investigated in all samples. Overall, BcVg1-like protein abundance increased as insects aged. However, lower abundance was observed in 7-day-old mated females compared to 7-day-old virgin females. It is important to highlight that mated females oviposited significantly more eggs than virgin females as was described in a previous study (Ibanez et al., 2017), and those eggs accumulate large amounts of Vg as vitellin. Significant differences were found in the comparison of 7-day-old Lso-free with LsoB- and LsoA- virgin females. Also, significant differences were observed when 7-day-old Lso-free mated females were compared with 7-day-old mated LsoB infected. No significant differences were found in comparisons of 1-day and 3-day-old virgin females (Lso-free, LsoA and LsoB).

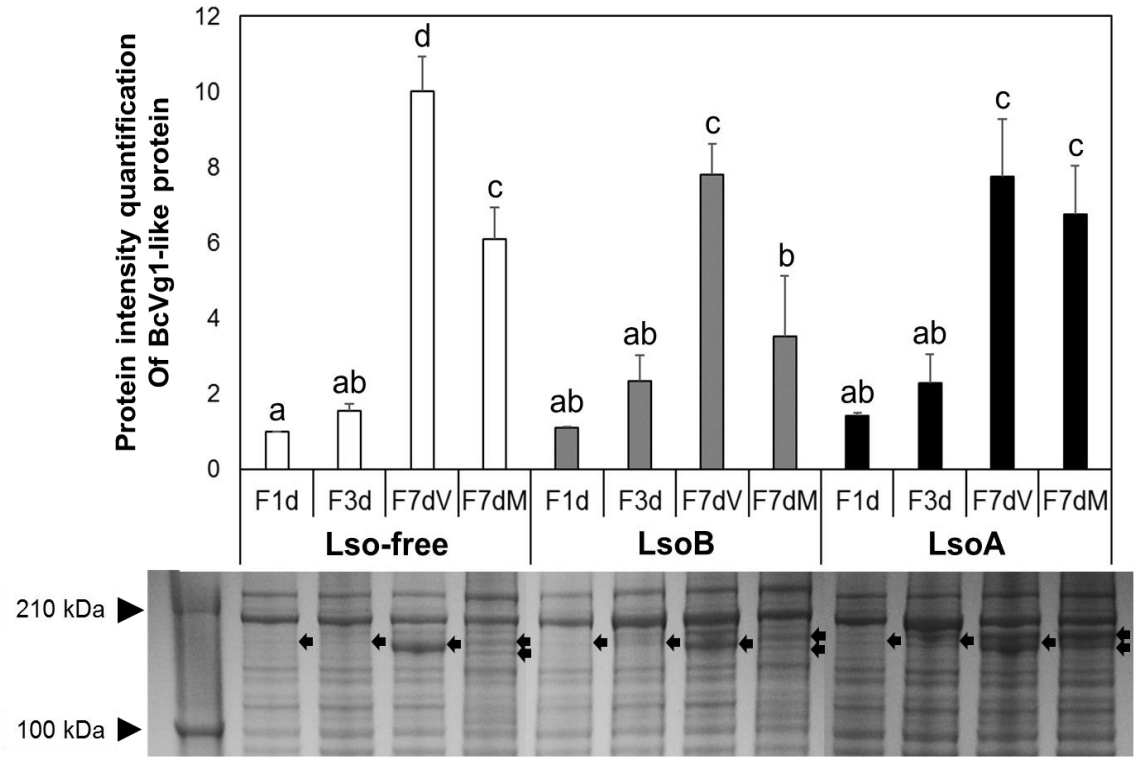

Figure 4.7. Vitellogenin protein expression analyses in *B. cockerelli* females Lso-free, LsoA and LsoB. Analysis of *B. cockerelli* proteins in different female life stages (20 µg per lane) by SDS-PAGE. Two high molecular weight abundant bands proteins ~200 kDa corresponding to BcVg1-like were highlighted with black arrows. 7-day-old LsoB-infected mated females had a reduced level of BcVg1-like protein compared to Lso-free and LsoA-infected females. This experiment was repeated three independent times. ST represents the protein molecular weight standard. Different letters indicate statistical differences between treatments using one-way ANOVA with Tukey's post hoc test ($P < 0.05$).

4.5 Discussion

This study tested the hypothesis that Lso-infection negatively affects the reproduction of *B. cockerelli* by reducing the transcript expression of genes potentially involved in vitellogenesis including: *BcVg1-like, Krüppel homolog 1-like, S6 kinase, Juvenile hormone esterase and juvenile hormone epoxide hydrolase*.

In a previous study, *BcVg1-like* was associated with psyllid reproduction suggesting that this protein provides the energy reserves for developing embryos (Ibanez et al., 2017). However, Vgs have been also associated with other biological functions. For instance, in immune defense in non-mammalian vertebrates and invertebrates, cited in (Zhang et al., 2011). Vg from fish *Hexagrammos otakii,* was showed to bind to lipopolysaccharides from Gram-negative bacteria, lipoteichoic acids from Gram-positive bacteria, peptidoglycans from both Gram-positive and Gram-negative bacteria, β-1,3-glucans from eukaryotic fungi and laminarin from brown algae (Li et al., 2008). Whereas, in *Bombyx mori*, Vg protein could bind the Gram negative bacterium *Escherichia coli* and the Gram positive bacterium *Bacillus subtilis* which had a strong antibacterial activity (Singh et al., 2013). In *Anopheles gambiae,* Vg was able to interfere with the anti-Plasmodium response (Rono et al., 2010). These findings suggest that Vg might be involved in the defense of host against microbes. However, this is a hypothesis that need to be examined in *B. cockerelli*.

Our results showed that mated LsoB-infected females had a significant reduction in the level of BcVg1-like transcript and protein expression (Figures 4.4A and 4.7). Whereas, mated LsoA-infected females did not show significant differences compared to

mated Lso-free females (uninfected). The negative effects of pathogen-infection in insect's reproduction have been showed in *A. gambiae*, *D. melanogaster*, *E. intermedius* and *T. oceanicus* (Ahmed and Hurd, 2006, Nystrand and Dowling, 2014, McNamara et al., 2014, Reaney and Knell, 2010). For instance, in *A. gambiae*, the transcription of Vg, the Vg protein concentration in the hemolymph and Vitellin accumulation in oocytes, were reduced following the infection with *Plasmodium yoelii nigeriensis*, when ookinetes invaded the female's mosquito midguts (Ahmed et al., 2001). Also, when *A. gambiae* was challenged with an immune elicitor, lipopolysaccharide, a significant decrease in the accumulation of proteins were observed in the ovaries, also a reduction of eggs oviposited was characteristic in these mosquito's species (Ahmed et al., 2002). In *D. melanogaster* a reduced fecundity was observed during an acute phase of infection of a naturally occurring Gram-negative pathogen, *Providencia rettgeri* (McKean et al., 2008).

Reproduction in insects is a complex biological process because involves many factors including sensory receptors, nutrition, a proper signal integration in the brain by neuropeptides and the biosynthesis and metabolism of hormones. In *B. cockerelli*, as well other hemipterans such as *Pyrrhocoris apterus*, *Lethocerus deyrollei*, *Nilaparvata lugens* and *Riptortus clavatus* JH controls vitellogenesis (Shinoda et al., 1996, Nagaba et al., 2010, Nagaba et al., 2011, Smykal et al., 2014, Ibanez et al., 2017).

While we showed that JH regulates *BcVg1-like* in *B. cockerelli*, no association between the expression of genes involved in JH degradation and *BcVg1-like* was found. While no transcriptional changes of JH degradation genes were observed, differences in

the activity and/or the abundance of these proteins in *B. cockerelli* females might occur. Also, there were not changes in the expression of JH-degradation genes induced by the presence of Lso in *B. cockerelli* females. Similarly, differences in JH biosynthesis could lead to differences in JH titer among the females, however these were not assessed.

Nutrients are important signals regulating reproduction in insects (Smykal and Raikhel, 2015). Therefore, we measured the expression of a transcript involved in TOR pathway, *S6 kinase*. A study performed in the mosquito *Aedes aegypti* showed that the depletion of S6K transcript using RNAi, disrupted the expression of Vg and resulted in reduced number of maturing eggs in mosquito females (Hansen et al., 2005). Our study showed that S6K expression was not modified by Lso-infection, except for the higher expression observed in LsoA- and LsoB-infected 3-day-old females compared with Lso-free females. But, this gene was upregulated in 3- and 7-day old mated females compared to 1-day-old and 7-day-old virgin females. The low level of S6K gene expression observed in 7-day-old virgin females could be related to an arrested vitellogenic process, similar to the "previtellogenic period" observed in mosquito females before the blood meal. If the latter is occurring, our expression analyses would suggest that S6 kinase might be indispensable for a TOR-dependent activation of Vg synthesis and oocyte maturation in *B. cockerelli* mated females. Also, the nutritional status of the insect might be affected by status of plant health. The presence of the Lso pathogen in potato plants modified the plant primary and secondary metabolites (Wallis et al., 2012, Wallis et al., 2015), and these metabolites might influence the fitness of *B. cockerelli*, see review (Tamborindeguy et al., 2017).

During insect vitellogenesis, the transcription factors methoprene-tolerant and Krüppel homolog-1 (Kr-h1) are critical in some insect species (Smykal et al., 2014, Song et al., 2014, Gujar and Palli, 2016). However, the transcription factors involved in *B. cockerelli* vitellogenesis have not been clearly determined. For this study, the transcript of *Kr-h1* was identified in a transcriptome database (Nachappa et al., 2012a), and our results suggest that Kr-h1 might have a role in psyllid vitellogenesis. This can be inferred from the pattern of expression of *Kr-h1* and *Vg*, for which an upregulation was observed in 7-day-old mated females compared with the level of expression in 1-day and 3-day-old females. Also, the expression of *Kr-h1* in 7-day-old Lso-free and LsoA mated females was higher than the relative expression observed in 7-day-old LsoB mated females. These results suggest that LsoB-infection results in a reduced expression of *Kr-h1*. *Kr-h1* reduction could be associated with the reduction of Vg (transcript and protein) expression in mated females resulting in a reduced reproductive output. The association of Kr-h1 and Vg expression in insect vitellogenic process has been previously described in several insect species. For example, in females of *Locusta migratoria*, a knockdown of *Kr-h1* reduces the *Vg* expression in the fat body. In that study, a reduction in oocyte maturation and ovarian growth were observed also (Song et al., 2014). However, in *Pyrrhocoris apterus* females the Kr-h1 RNAi did not have an impact on *Vg* expression or ovarian development (Smykal et al., 2014). Similarly, in the common bed bug, *Cimex lectularius*, the knockdown of *Kr-h1* in adult females did not reduce their fecundity but affected the development of embryos in the eggs laid by females (Gujar and Palli, 2016). Thus, to understand in detail the putative roles of Kr-h1 in *B. cockerelli* vitellogenesis,

more refined experiments need to be performed, such as the use of double strand RNA (dsRNA).

In this study, it was clear that both Lso haplotypes had different effects on several aspects of *B. cockerelli* females' reproduction. These differences between Lso A and Lso B could arise from genetic differences between these two haplotypes such as gain/loss of genes or be the consequence of the genomic rearrangements that result in changes of transcript expression in pathogenesis-related genes, as was proposed in (Yao et al., 2016). These differences in gene expression might be implicated in pathogenicity and an activation of the insect immune system could reduce the reproductive output, as was the outcome in this study. This follows the idea of the resource allocation model in which a the trade-off arises because of competition for one or more limiting resources (Schwenke et al., 2016). Also, it is possible that LsoB may disrupt multiple stages of vitellogenesis, and/or initiate an ovarian pathology that feeds back negatively the fat body.

In conclusion, this is the first study performed in *B. cockerelli* that showed changes in transcript expression of genes involved in vitellogenesis in LsoA- and LsoB-infected females. Also, the reduced pathogenicity of LsoA compared with LsoB might be an advantage enhancing the probability of LsoA transmission, because of the comparable level of oviposition between Lso-free and LsoA females. However, LsoB might have an adaptive strategy in which *B. cockerelli* benefits from it under specific environmental circumstances and not related to its diminished reproduction, and this interaction might need to be investigated in detail.

5. CONCLUSIONS AND FUTURE DIRECTIONS

The potato psyllid, *Bactericera cockerelli* (Šulc) (Hemiptera: Triozidae), is a phloem-feeding insect with preference for *Solanaceae*. This insect species is vector of the pathogenic bacteria '*Candidatus* Liberibacter solanacearum' the causative agents of zebra chip, an important disease of commercial potatoes in several countries worldwide. The recent classification of psyllids among the most dangerous vectors has promoted their study, but still many biological processes such as reproduction and vitellogenesis need to be investigated. The vitellogenic process (vitellogenesis) has been studied in many insect species and the reproductive success of all oviparous species depends on Vitellogenin (Vg) biosynthesis and its accumulation in the developing oocytes, as vitellin. However, the knowledge of this biological process in phloem feeder hemipterans is still limited. In fact, when we began the study of vitellogenesis in *B. cockerelli*, only one Vg mRNA was identified in a member of the Sternorrhyncha suborder, *Bemisia tabaci* (Guo et al., 2012) . New studies were performed in this research area during my dissertation; but, those studies were focused in other insect species that did not belong to de Sternorrhyncha suborder.

This is the first study in *B. cockerelli* that focused in the molecular aspects of reproduction. We identified several genes that might be involved directly and indirectly with reproduction, among them two vitellogenins (*BcVg1-like* and *BcVg6-like*), a transcription factor involved in the vitellogenic process in other hemipterans, *Krüppel homolog1*, two genes involve in juvenile hormone (JH) degradation *JH esterase* and *JH*

epoxide hydrolase and a gene involved in the Target of rapamycin (TOR) pathway, *S6 Kinase*.

This work represents an important leap in our understanding of *B. cockerelli* reproduction. Based on Bayesian inferences and *in-silico* predictions of the domain structures of each Vg I showed that one of the identified Vg genes is a conventional Vg protein (BcVg1-like) that conserved its ancestral function as an egg yolk-precursor (Chapter 2) while the other Vg gene (*BcVg6-like*) might not be directly involved in reproduction and might have acquired a new and still undetermined function in *B. cockerelli* (Chapter 3). I also determined that *BcVg1-like* had a female-biased expression and it was highly expressed after mating and exogenous applications of JH III (Chapter 2) This is a first step towards understanding the hormonal control of vitellogenesis in *B. cockerelli*. I also showed that exogenous applications of JH III induced the production of developing oocytes in *B. cockerelli* virgin females. This result could suggest that JH III not only controls the vitellogenin synthesis; but, also could be involve in other processes such as the upregulation of *Vitellogenin receptor* and/or its translocation to the surface of oocytes, facilitating the uptake of Vg during its development in *B. cockerelli* females. Future experiments could be aimed at identifying the Vg receptor in *B. cockerelli* and studying its expression and localization in virgin and mated females. Also, whether its expression and localization is regulated by JH needs to be investigated.

Finally, I determined that LsoB-infected females had a significant reduction in the level of BcVg1-like transcript and protein expression. However, mated LsoA-infected females did not show significant differences compared to mated uninfected

females. Similar expression profile was found for *Kr-h1*. The association between Kr-h1 and Vg expression in the insect vitellogenic process has been previously described in other insect species. Thus, to understand in detail this relationship in *B. cockerelli* vitellogenesis, more refined experiments need to be performed, such as the use of double strand RNA (dsRNA) to generate 'knock-out' phenotypes preventing the target gene expression, and finally elucidate the function of these target genes.

In summary, my analyses showed clearly that both Lso haplotypes had different effects on several aspects of *B. cockerelli* females' reproduction. The reduced pathogenicity of LsoA compared with LsoB might be an advantage enhancing the probability of LsoA transmission. However, LsoB might have an adaptive strategy in which *B. cockerelli* benefits from it under specific environmental circumstances and not related to its diminished reproduction. These interactions need to be investigated in more detail.

Overall, the results presented in this dissertation help us to proposed a putative vitellogenesis model in *B. cockerelli* (Figure 5.1). Based on my results, I also propose to divide the vitellogenesis in this insect species into a previtellogenic period and a vitellogenic period (Figure 5.2). During the previtellogenic period, which begins after the molting from nymph to adult, the female fat body prepares the transcriptional and translational molecular machineries necessary for vitellogenin synthesis. The previtellogenic period finishes after mating. I speculate that following mating there is a burst in the level of JH, produced by the corpora allata. This JH burst triggers in the fat body tissue the transcription and synthesis of vitellogenins, that are secreted to the

hemolymph and internalized via receptor-mediated endocytosis into the oocytes. The signal that triggers that JH burst following mating needs to be determined.

Indeed, to further advance our understanding of the molecular aspects of reproduction in *B. cockerelli*, we must determine how the brain transduces the 'mating' signal to induce vitellogenesis. In other insect species, the main neuropeptides involved in this signal transduction are allatropin and allatostanin which regulate the synthesis and release of JH by the corpora allata, inducing or reducing the level of JH in the hemolymph. An experiment that might help us to explore the nervous system is using mass spectrometry-based peptidomes.

This study was focused on the expression of candidate genes during vitellogenesis in *B. cockerelli*. These genes were chosen because they are involved in this biological process in other insect species. However, to advance and fully understand the function of these putative proteins, it will be necessary to change and perform new approaches. For example, specific antibodies for each protein need to be generated, this is essential because it will permit us to do western blots and immunofluorescence assays, indicating where these proteins are located (which tissue) and understand the dynamic between the expression of the transcript and the translation of the protein. These results will complement our results concerning the functions of these proteins during the vitellogenesis in *B. cockerelli* females, and certainly will result on new questions.

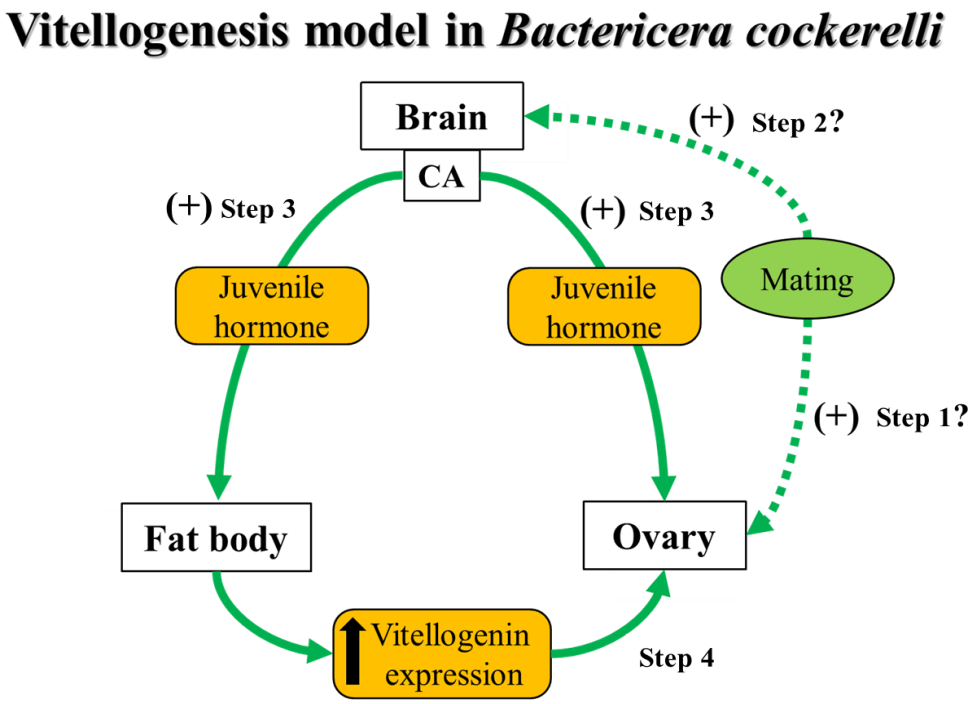

Figure 5.1. Proposed vitellogenesis model in *B. cockerelli*. During vitellogenesis several processes are occurring, mating can trigger Step 1 and 2, is still unknown in *B. cockerelli*, but in other insect species it has been proposed that some sperm activating substances (Juvenile hormones, proteins and peptides) are and transferred to females during mating, and these substances induce the ovary development (**Step 1**) and/or the stimulate the brain to induce the JH synthesis in the corpora allata and posterior release to the hemolymph (**Step 2**), when JH reach the female's tissues this hormone induces different responses (**Step 3**), in the ovary might be implicated in the expression and posterior translocation of Vitellogenin receptor to the membrane of the oocytes (undetermined in *B. cockerelli*) and in the fat body JH stimulates the synthesis of Vitellogenin (BcVg1-like). During the last step (**Step 4**) the synthetized vitellogenin protein is deposited into the oocytes trough a receptor-mediated endocytosis.

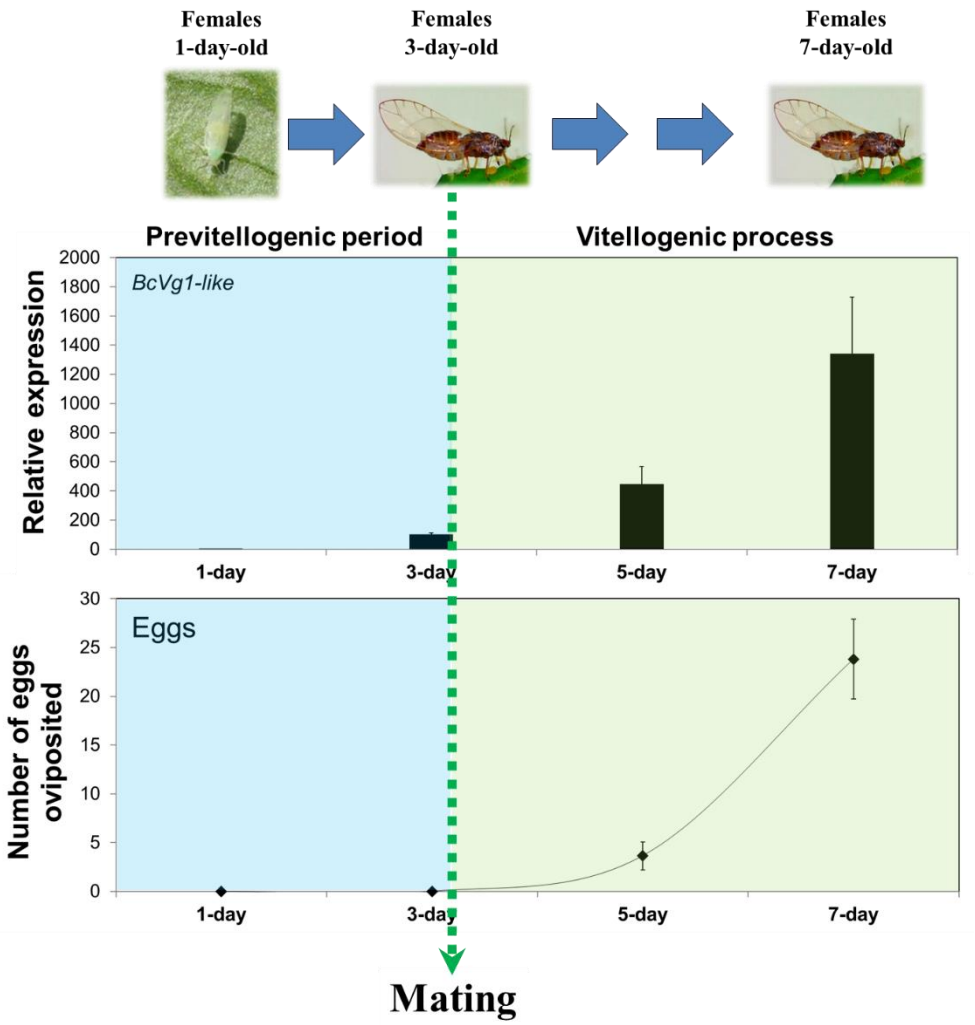

Figure 5.2. Schematic representation of *B. cockerelli* vitellogenesis. The results observed in this study help us to propose that vitellogenesis in females can be divided in two periods, the **previtellogenic period** (PVP) and the **vitellogenic period** (VP). In PVP, changes at transcriptional and translational level are occurring in females that allow the fat body cells have the machinery necessary for Vg synthesis. After, mating and during the VP large amount of vitellogenin protein is synthetized in the fat body cells and all this protein is being transported and deposited in the developing oocytes in *B. cockerelli*.

REFERENCES

ABDULLAH, N. 2008. Life history of the potato psyllid *Bactericera cockerelli* (Homoptera: Psyllidae) in controlled environment agriculture in Arizona. *African Journal of Agricultural Research,* 3**,** 060-067.

AHMED, A., BAGGOTT, S., MAINGON, R. & HURD, H. 2002. The costs of mounting an immune response are reflected in the reproductive fitness of the mosquito Anopheles gambiae. *Oikos,* 97**,** 371-377.

AHMED, A., MAINGON, R., ROMANS, P. & HURD, H. 2001. Effects of malaria infection on vitellogenesis in Anopheles gambiae during two gonotrophic cycles. *Insect molecular biology,* 10**,** 347-356.

AHMED, A. M. & HURD, H. 2006. Immune stimulation and malaria infection impose reproductive costs in Anopheles gambiae via follicular apoptosis. *Microbes and Infection,* 8**,** 308-315.

ALTSCHUL, S. F., GISH, W., MILLER, W., MYERS, E. W. & LIPMAN, D. J. 1990. Basic local alignment search tool. *J Mol Biol,* 215**,** 403-410.

ARNETT JR, R. H. 2000. *American insects: a handbook of the insects of America north of Mexico,* Crc Press.

ARSIC, D. & GUERIN, P. M. 2008. Nutrient content of diet affects the signaling activity of the insulin/target of rapamycin/p70 S6 kinase pathway in the African

malaria mosquito Anopheles gambiae. *Journal of insect physiology,* 54**,** 1226-1235.

ARUKWE, A. & GOKSØYR, A. 2003. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. *Comp Hepatol,* 2**,** 1.

AUBERT, B. Citrus greening disease, a serious limiting factor for citriculture in Asia and Africa. *Proceedings of the International Society of Citriculture,* 1992. 817-820.

BELLES, X. Endocrine effectors in insect vitellogenesis. Seminar series-society for experimental biology, 1998. Cambridge University Press, 71-90.

BLANK, S., SEISMANN, H., MCINTYRE, M., OLLERT, M., WOLF, S., BANTLEON, F. I. & SPILLNER, E. 2013. Vitellogenins are new high molecular weight components and allergens (Api m 12 and Ves v 6) of *Apis mellifera* and *Vespula vulgaris* venom. *PLoS One,* 8**,** e62009.

BLOCH, G., HEFETZ, A. & HARTFELDER, K. 2000. Ecdysteroid titer, ovary status, and dominance in adult worker and queen bumble bees (*Bombus terrestris*). *Journal of Insect Physiology,* 46**,** 1033-1040.

BOVÉ, J. M. 2006. Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. *Journal of plant pathology***,** 7-37.

CAMERON, S. L., BECKENBACH, A. T., DOWTON, M. P. & WHITING, M. F. 2006. Evidence from mitochondrial genomics on interordinal relationships in insects. *Arthropod Sytematics & Phylogeny*, 64**,** 27-34.

CHEN, J.-S., SAPPINGTON, T. W. & RAIKHEL, A. S. 1997. Extensive sequence conservation among insect, nematode, and vertebrate vitellogenins reveals ancient common ancestry. *Journal of Molecular Evolution,* 44**,** 440-451.

CHENG, D., MENG, M., PENG, J., QIAN, W., KANG, L. & XIA, Q. 2014. Genome-wide comparison of genes involved in the biosynthesis, metabolism, and signaling of juvenile hormone between silkworm and other insects. *Genetics and molecular biology,* 37**,** 444-459.

COMAS, D., PIULACHS, M. & BELLÉS, X. 1999. Fast induction of vitellogenin gene expression by juvenile hormone III in the cockroach *Blattella germanica* (L.)(Dictyoptera, Blattellidae). *Insect biochemistry and molecular biology,* 29**,** 821-827.

CROSSLIN, J. M., MUNYANEZA, J. E., BROWN, J. K. & LIEFTING, L. W. 2010. Potato zebra chip disease: a phytopathological tale. *Plant Health Progress,* 17.

CRYAN, J. R. & URBAN, J. M. 2012. Higher-level phylogeny of the insect order Hemiptera: is Auchenorrhyncha really paraphyletic? *Systematic Entomology,* 37**,** 7-21.

DAVEY, K. G. 1993. Hormonal integration of egg-production in *Rhodnius prolixus*. *American Zoologist,* 33**,** 397-402.

DE BARRO, P. J., LIU, S.-S., BOYKIN, L. M. & DINSDALE, A. B. 2011. *Bemisia tabaci*: a statement of species status. *Annual review of entomology,* 56**,** 1-19.

DELBECQUE , J.-P., WEIDNER, K. & HOFFMANN, K. H. 1990. Alternative sites for ecdysteroid production in insects. *Invertebrate Reproduction & Development,* 18**,** 29-42.

DHADIALLA, T. & RAIKHEL, A. 1994. Endocrinology of mosquito vitellogenesis. *Perspectives in comparative endocrinology***,** 275-281.

DHADIALLA, T. S., HAYS, A. R. & RAIKHEL, A. S. 1992. Characterization of the solubilized mosquito vitellogenin receptor. *Insect biochemistry and molecular biology,* 22**,** 803-816.

DOLLING, W. R. 1991. *Hemiptera*, Oxford University Press.

DON-WHEELER, G. & ENGELMANN, F. 1991. The female-and male-produced vitellogenins of *Leucophaea maderae*. *Journal of insect physiology,* 37**,** 869-882.

DOTSON, E. & BEARD, C., 3 2001. Sequence and organization of the mitochondrial genome of the Chagas disease vector, *Triatoma dimidiata*. *Insect molecular biology,* 10**,** 205-215.

ENGELMANN, F. 1983. Vitellogenesis controlled by juvenile hormone. *Endocrinology of insects,* 1**,** 259-270.

ENGELMANN, F. 2002. Ecdysteroids, juvenile hormone and vitellogenesis in the cockroach *Leucophaea maderae*. *Journal of Insect Science,* 2**,** 20.

FESCEMYER, H. W., MASLER, E. P., DAVIS, R. E. & KELLY, T. J. 1992. Vitellogenin synthesis in female larvae of the gypsy moth, *Lymantria dispar* (L.): suppression by juvenile hormone. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* 103**,** 533-542.

FRIESEN, K. J. & KAUFMAN, W. R. 2004. Effects of 20-hydroxyecdysone and other hormones on egg development, and identification of a vitellin-binding protein in the ovary of the tick, *Amblyomma hebraeum*. *Journal of insect physiology,* 50**,** 519-529.

GERBER-HUBER, S., NARDELLI, D., HAEFLIGER, J.-A., COOPER, D. N., GIVEL, F., GERMOND, J.-E., ENGEL, J., GREEN, N. M. & WAHLI, W. 1987. Precursor-product relationship between vitellogenin and the yolk proteins as derived from the complete sequence of a *Xenopus* vitellogenin gene. *Nucleic acids research,* 15**,** 4737-4760.

GOULAS, A., TRIPLETT, E. & TABORSKY, G. 1996. Oligophosphopeptides of varied structural complexity derived from the egg phosphoprotein, phosvitin. *Journal of protein chemistry,* 15**,** 1-9.

GUIDUGLI, K. R., NASCIMENTO, A. M., AMDAM, G. V., BARCHUK, A. R., OMHOLT, S., SIMÕES, Z. L. & HARTFELDER, K. 2005. Vitellogenin regulates hormonal dynamics in the worker caste of a eusocial insect. *FEBS letters,* 579**,** 4961-4965.

GUJAR, H. & PALLI, S. R. 2016. Juvenile hormone regulation of female reproduction in the common bed bug, Cimex lectularius. *Scientific reports,* 6.

GUO, J.-Y., DONG, S.-Z., YANG, X.-L., CHENG, L., WAN, F.-H., LIU, S.-S., ZHOU, X.-P. & YE, G.-Y. 2012. Enhanced vitellogenesis in a whitefly via feeding on a begomovirus-infected plant. *PloS one,* 7**,** e43567.

HAGEDORN, H., O'CONNOR, J., FUCHS, M. S., SAGE, B., SCHLAEGER, D. A. & BOHM, M. 1975. The ovary as a source of alpha-ecdysone in an adult mosquito. *Proceedings of the National Academy of Sciences,* 72**,** 3255-3259.

HANSEN, A., TRUMBLE, J., STOUTHAMER, R. & PAINE, T. 2008. A new huanglongbing species,"Candidatus Liberibacter psyllaurous," found to infect tomato and potato, is vectored by the psyllid Bactericera cockerelli (Sulc). *Applied and environmental microbiology,* 74**,** 5862-5865.

HANSEN, I. A., ATTARDO, G. M., ROY, S. G. & RAIKHEL, A. S. 2005. Target of rapamycin-dependent activation of S6 kinase is a central step in the transduction of nutritional signals during egg development in a mosquito. *Journal of Biological Chemistry,* 280**,** 20565-20572.

HARSHMAN, L. G., LOEB, A. M. & JOHNSON, B. A. 1999. Ecdysteroid titers in mated and unmated *Drosophila melanogaster* females. *Journal of insect physiology,* 45**,** 571-577.

HARTFELDER, K. & EMLEN, D. 2012. Endocrine control of insect polyphenism. *Insect Endocrinology***,** ed. LI Gilbert, pp. 464–522. Boston: Elsevier.

HAVUKAINEN, H., MÜNCH, D., BAUMANN, A., ZHONG, S., HALSKAU, Ø., KROGSGAARD, M. & AMDAM, G. V. 2013. Vitellogenin recognizes cell damage through membrane binding and shields living cells from reactive oxygen species. *J. Biol Chem,* 288**,** 28369-28381.

HERNÁNDEZ-MARTÍNEZ, S., MAYORAL, J. G., LI, Y. & NORIEGA, F. G. 2007. Role of juvenile hormone and allatotropin on nutrient allocation, ovarian development and survivorship in mosquitoes. *Journal of insect physiology,* 53**,** 230-234.

HORIGANE, M., SHINODA, T., HONDA, H. & TAYLOR, D. 2010. Characterization of a vitellogenin gene reveals two phase regulation of vitellogenesis by engorgement and mating in the soft tick *Ornithodoros moubata* (Acari: Argasidae). *Insect molecular biology,* 19**,** 501-515.

HUELSENBECK, J. P. & RONQUIST, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics,* 17**,** 754-755.

IBANEZ, F., HANCOCK, J. & TAMBORINDEGUY, C. 2014. Identification and expression analysis of aquaporins in the potato psyllid, *Bactericera cockerelli*. *PloS one,* 9**,** e111745.

IBANEZ, F., LEVY, J. & TAMBORINDEGUY, C. 2017. Identification and expression analyses of vitellogenin in *Bactericera cockerelli* (Šulc). *Journal of insect physiology,* 98**,** 205-213.

IBANEZ, F. & TAMBORINDEGUY, C. 2016. Selection of reference genes for expression analysis in the potato psyllid, *Bactericera cockerelli*. *Insect molecular biology*.

INNAN, H. & KONDRASHOV, F. 2010. The evolution of gene duplications: classifying and distinguishing between models. *Nat Rev Genet,* 11**,** 97-108.

JACKSON, B. C., GOOLSBY, J., WYZYKOWSKI, A., VITOVSKY, N. & BEXTINE, B. 2009. Analysis of genetic relationships between potato psyllid (*Bactericera cockerelli*) populations in the United States, Mexico and Guatemala using ITS2 and Inter Simple Sequence Repeat (ISSR) data. *Plant Sci,* 61**,** 1-5.

KATAOKA, H., TOSCHI, A., LI, J. P., CARNEY, R. L., SCHOOLEY, D. A. & KRAMER, S. J. 1989. Identification of an allatotropin from adult Manduca sexta. *Science,* 243**,** 1481.

KELLY, T. J. & HUNT, L.-M. 1982. Endocrine influence upon the development of vitellogenic competency in *Oncopeltus fasciatus*. *Journal of Insect Physiology,* 28**,** 935-941.

KOCHER, S. D., RICHARD, F.-J., TARPY, D. R. & GROZINGER, C. M. 2008. Genomic analysis of post-mating changes in the honey bee queen (*Apis mellifera*). *BMC genomics,* 9**,** 1.

LAFONT, R., DAUPHIN-VILLEMANT, C., WARREN, J. & REES, H. 2012. Ecdysteroid chemistry and biochemistry. *Insect Endocrinology***,** 106-176.

LEE, J. M., HATAKEYAMA, M. & OISHI, K. 2000a. A simple and rapid method for cloning insect vitellogenin cDNAs. *Insect biochemistry and molecular biology,* 30**,** 189-194.

LEE, J. M., NISHIMORI, Y., HATAKEYAMA, M., BAE, T.-W. & OISHI, K. 2000b. Vitellogenin of the cicada *Graptopsaltria nigrofuscata* (Homoptera): analysis of its primary structure. *Insect biochemistry and molecular biology,* 30**,** 1-7.

LI, Z., ZHANG, S. & LIU, Q. 2008. Vitellogenin functions as a multivalent pattern recognition receptor with an opsonic activity. *PLoS One,* 3**,** e1940.

LIEFTING, L., PEREZ-EGUSQUIZA, Z., CLOVER, G. & ANDERSON, J. 2008. A new '*Candidatus* Liberibacter'species in *Solanum tuberosum* in New Zealand. *Plant Dis,* 92**,** 1474-1474.

LIN, H., ISLAM, M. S., BAI, Y., WEN, A., LAN, S., GUDMESTAD, N. C. & CIVEROLO, E. L. 2012. Genetic diversity of ‘Cadidatus Liberibacter solanacearum’strains in the United States and Mexico revealed by simple sequence repeat markers. *European Journal of Plant Pathology,* 132**,** 297-308.

LIU, D. & TRUMBLE, J. T. 2007. Comparative fitness of invasive and native populations of the potato psyllid (*Bactericera cockerelli*). *Entomologia experimentalis et applicata,* 123**,** 35-42.

LOEB, M. J., DE LOOF, A., GELMAN, D. B., HAKIM, R. S., JAFFE, H., KOCHANSKY, J. P., MEOLA, S. M., SCHOOFS, L., STEEL, C. & VAFOPOULOU, X. 2001. Testis ecdysiotropin, an insect gonadotropin that induces synthesis of ecdysteroid. *Archives of insect biochemistry and physiology,* 47**,** 181-188.

LONG, M., BETRÁN, E., THORNTON, K. & WANG, W. 2003. The origin of new genes: glimpses from the young and old. *Nat Rev Genet,* 4**,** 865-875.

MANJUNATH, K., HALBERT, S., RAMADUGU, C., WEBB, S. & LEE, R. 2008. Detection of '*Candidatus* Liberibacter asiaticus' in *Diaphorina citri* and its importance in the management of citrus huanglongbing in Florida. *Phytopathology,* 98**,** 387-396.

MARCHLER-BAUER, A., DERBYSHIRE, M. K., GONZALES, N. R., LU, S., CHITSAZ, F., GEER, L. Y., GEER, R. C., HE, J., GWADZ, M. & HURWITZ,

D. I. 2014. CDD: NCBI's conserved domain database. *Nucleic acids research***,** gku1221.

MCKEAN, K. A., YOURTH, C. P., LAZZARO, B. P. & CLARK, A. G. 2008. The evolutionary costs of immunological maintenance and deployment. *BMC evolutionary biology,* 8**,** 76.

MCNAMARA, K., LIESHOUT, E. & SIMMONS, L. 2014. Females suffer a reduction in the viability of stored sperm following an immune challenge. *Journal of evolutionary biology,* 27**,** 133-140.

MENON, A., VARMA, V. & SHARMA, V. K. 2014. Rhythmic egg-laying behaviour in virgin females of fruit flies *Drosophila melanogaster*. *Chronobiology international,* 31**,** 433-441.

MONTEIRO, F. A., BARRETT, T. V., FITZPATRICK, S., CORDON-ROSALES, C., FELICIANGELI, D. & BEARD, C. B. 2003. Molecular phylogeography of the Amazonian Chagas disease vectors *Rhodnius prolixus* and *R. robustus*. *Molecular Ecology,* 12**,** 997-1006.

MORANDIN, C., HAVUKAINEN, H., KULMUNI, J., DHAYGUDE, K., TRONTTI, K. & HELANTERÄ, H. 2014. Not only for egg yolk-functional and evolutionary insights from expression, selection and structural analyses of *Formica* ant vitellogenins. *Mol Biol Evol***,** msu171.

MOSHITZKY, P. & MORIN, S. 2014. *Bemisia tabaci* females from the Mediterranean (Q) species detect and avoid laying eggs in the presence of pyriproxyfen, a juvenile hormone analogue. *Pest management science,* 70**,** 1468-1476.

MOUCHEL, N., TRICHET, V., BETZ, A., LE PENNEC, J.-P. & WOLFF, J. 1996. Characterization of vitellogenin from rainbow trout (*Oncorhynchus mykiss*). *Gene,* 174**,** 59-64.

MÜNCH, D. & AMDAM, G. V. 2010. The curious case of aging plasticity in honey bees. *FEBS lett,* 584**,** 2496-2503.

MUNYANEZA, J., CROSSLIN, J. & UPTON, J. 2007a. Association of Bactericera cockerelli (Homoptera: Psyllidae) with "zebra chip," a new potato disease in southwestern United States and Mexico. *Journal of Economic Entomology,* 100**,** 656-663.

MUNYANEZA, J. E. 2012. Zebra chip disease of potato: biology, epidemiology, and management. *Am J Potato Res,* 89**,** 329-350.

MUNYANEZA, J. E., CROSSLIN, J. M. & BUCHMAN, J. L. 2009. Seasonal occurrence and abundance of the potato psyllid, *Bactericera cockerelli*, in south central Washington. *American journal of potato research,* 86**,** 513-518.

MUNYANEZA, J. E., FISHER, T. W., SENGODA, V. G., GARCZYNSKI, S. F., NISSINEN, A. & LEMMETTY, A. 2010. Association of "*Candidatus*

Liberibacter solanacearum" with the psyllid, *Trioza apicalis* (Hemiptera: Triozidae) in Europe. *J Econ Entomol,* 103**,** 1060-1070.

MUNYANEZA, J. E., GOOLSBY, J. A., CROSSLIN, J. M. & UPTON, J. E. 2007b. Further evidence that zebra chip potato disease in the lower Rio Grande Valley of Texas is associated with Bactericera cockerelli. *Subtropical Plant Science,* 59**,** 30-37.

NACHAPPA, P., LEVY, J., PIERSON, E. & TAMBORINDEGUY, C. 2014. Correlation between "Candidatus Liberibacter solanacearum" infection levels and fecundity in its psyllid vector. *Journal of invertebrate pathology,* 115**,** 55-61.

NACHAPPA, P., LEVY, J. & TAMBORINDEGUY, C. 2012a. Transcriptome analyses of *Bactericera cockerelli* adults in response to '*Candidatus* Liberibacter solanacearum' infection. *Molecular genetics and genomics,* 287**,** 803-817.

NACHAPPA, P., SHAPIRO, A. A. & TAMBORINDEGUY, C. 2012b. Effect of 'Candidatus Liberibacter solanacearum'on fitness of its insect vector, Bactericera cockerelli (Hemiptera: Triozidae), on tomato. *Phytopathology,* 102**,** 41-46.

NAGABA, Y., TUFAIL, M., INUI, H. & TAKEDA, M. 2010. Hormonal regulation and effects of four environmental polutants on vitellogenin gene transcription in the giant water bug, *Lethocerus deyrollei* (Heteroptera: Belostomatidae). *J Insect Conserv*.

NAGABA, Y., TUFAIL, M., INUI, H. & TAKEDA, M. 2011. Hormonal regulation and effects of four environmental pollutants on vitellogenin gene transcription in the giant water bFfigug, Lethocerus deyrollei (Hemiptera: Belostomatidae). *Journal of insect conservation,* 15**,** 421-431.

NELSON, C. M., IHLE, K. E., FONDRK, M. K., PAGE JR, R. E. & AMDAM, G. V. 2007. The gene vitellogenin has multiple coordinating effects on social organization. *PLoS biology,* 5**,** e62.

NELSON, W. R., FISHER, T. W. & MUNYANEZA, J. E. 2011. Haplotypes of "Candidatus Liberibacter solanacearum" suggest long-standing separation. *European Journal of Plant Pathology,* 130**,** 5-12.

NELSON, W. R., SENGODA, V. G., ALFARO-FERNANDEZ, A. O., FONT, M. I., CROSSLIN, J. M. & MUNYANEZA, J. E. 2013. A new haplotype of "Candidatus Liberibacter solanacearum" identified in the Mediterranean region. *European Journal of Plant Pathology,* 135**,** 633-639.

NIJHOUT, H. F. 2013. Arthropod developmental endocrinology. *Arthropod Biology and Evolution.* Springer.

NORIEGA, F., RIBEIRO, J., KOENER, J., VALENZUELA, J., HERNANDEZ-MARTINEZ, S., PHAM, V. & FEYEREISEN, R. 2006. Comparative genomics of insect juvenile hormone biosynthesis. *Insect biochemistry and molecular biology,* 36**,** 366-374.

NORIEGA, F. G. 2014. Juvenile hormone biosynthesis in insects: what is new, what do we know, and what questions remain? *International Scholarly Research Notices,* 2014.

NYSTRAND, M. & DOWLING, D. 2014. Dose-dependent effects of an immune challenge at both ultimate and proximate levels in Drosophila melanogaster. *Journal of evolutionary biology,* 27**,** 876-888.

OGIHARA, K., HORIGANE, M., NAKAJIMA, Y., MORIBAYASHI, A. & TAYLOR, D. 2007. Ecdysteroid hormone titer and its relationship to vitellogenesis in the soft tick, *Ornithodoros moubata* (Acari: Argasidae). *General and comparative endocrinology,* 150**,** 371-380.

PÉREZ-HEDO, M., RIVERA-PEREZ, C. & NORIEGA, F. G. 2013. The insulin/TOR signal transduction pathway is involved in the nutritional regulation of juvenile hormone synthesis in Aedes aegypti. *Insect biochemistry and molecular biology,* 43**,** 495-500.

PRATT, G. & DAVEY, K. 1972. The corpus allatum and oogenesis in *Rhodnius prolixus* (Stål.). *Journal of experimental biology,* 56**,** 201-214.

PRINCE, V. E. & PICKETT, F. B. 2002. Splitting pairs: the diverging fates of duplicated genes. *Nat Rev Genet,* 3**,** 827-837.

RAIKHEL, A., BROWN, M. & BELLES, X. 2005. Hormonal control of reproductive processes. *Comprehensive molecular insect science,* 3**,** 433-491.

RAIKHEL, A. S. 1992. Vitellogenesis in mosquitoes. *Advances in Disease Vector Research.* Springer.

RAIKHEL, A. S., KOKOZA, V. A., ZHU, J., MARTIN, D., WANG, S.-F., LI, C., SUN, G., AHMED, A., DITTMER, N. & ATTARDO, G. 2002. Molecular biology of mosquito vitellogenesis: from basic studies to genetic engineering of antipathogen immunity. *Insect biochemistry and molecular biology,* 32**,** 1275-1286.

RAMAKERS, C., RUIJTER, J. M., DEPREZ, R. H. L. & MOORMAN, A. F. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience letters,* 339**,** 62-66.

RAMBAUT, A. 2012. FigTree version 1.4. 0. *Available at ht tp://tree. bio. ed. ac. uk/software/figtree*.

RAUSHENBACH, I. Y., GRUNTENKO, N., BOWNES, M., ADONIEVA, N., TERASHIMA, J., KARPOVA, E., FADDEEVA, N. & CHENTSOVA, N. 2004. The role of juvenile hormone in the control of reproductive function in *Drosophila virilis* under nutritional stress. *Journal of insect physiology,* 50**,** 323-330.

REANEY, L. T. & KNELL, R. J. 2010. Immune activation but not male quality affects female current reproductive investment in a dung beetle. *Behavioral Ecology,* 21**,** 1367-1372.

REES, D. A., GILES, P., LEWIS, M. D. & HAM, J. 2010. Adenosine regulates thrombomodulin and endothelial protein C receptor expression in folliculostellate cells of the pituitary gland. *Purinergic signalling,* 6**,** 19-29.

RIDDIFORD, L. M. 1994. Cellular and molecular actions of juvenile hormone I. General considerations and premetamorphic actions. *In:* EVANS, P. D. (ed.) *Advances in Insect Physiology.* Academic Press.

RONO, M. K., WHITTEN, M. M., OULAD-ABDELGHANI, M., LEVASHINA, E. A. & MAROIS, E. 2010. The major yolk protein vitellogenin interferes with the anti-plasmodium response in the malaria mosquito Anopheles gambiae. *PLoS Biol,* 8**,** e1000434.

ROUILLÉ, Y., DUGUAY, S. J., LUND, K., FURUTA, M., GONG, Q., LIPKIND, G., OLIVA, A. A., CHAN, S. J. & STEINER, D. F. 1995. Proteolytic processing mechanisms in the biosynthesis of neuroendocrine peptides: the subtilisin-like proprotein convertases. *Frontiers in neuroendocrinology,* 16**,** 322-361.

SCHMITTGEN, T. D. & LIVAK, K. J. 2008. Analyzing real-time PCR data by the comparative CT method. *Nature protocols,* 3**,** 1101-1108.

SCHNEIDER, C. A., RASBAND, W. S. & ELICEIRI, K. W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature methods,* 9**,** 671.

SCHWENKE, R. A., LAZZARO, B. P. & WOLFNER, M. F. 2016. Reproduction–immunity trade-offs in insects. *Annual review of entomology,* 61**,** 239-256.

SECOR, G., RIVERA, V., ABAD, J., LEE, I.-M., CLOVER, G., LIEFTING, L., LI, X. & DE BOER, S. 2009. Association of '*Candidatus* Liberibacter solanacearum'with zebra chip disease of potato established by graft and psyllid transmission, electron microscopy, and PCR. *Plant Dis,* 93**,** 574-583.

SEEHUUS, S.-C., NORBERG, K., GIMSA, U., KREKLING, T. & AMDAM, G. V. 2006. Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *Proc Nat Acad Sci USA,* 103**,** 962-967.

SEHNAL, F. 1984. The juvenile hormone of insects. *Nova Acta Leopold,* 255**,** 251-266.

SHINODA, T., MIURA, K., TAYLOR, D. & CHINZEI, Y. 1996. Vitellogenins and vitellins in the bean bug, *Riptortus clavatus* (Hemiptera: Alydidae): purification, immunological identification, and induction by juvenile hormone. *Archives of insect biochemistry and physiology,* 31**,** 395-412.

SINGH, N. K., PAKKIANATHAN, B. C., KUMAR, M., PRASAD, T., KANNAN, M., KÖNIG, S. & KRISHNAN, M. 2013. Vitellogenin from the silkworm, Bombyx mori: an effective anti-bacterial agent. *PloS one,* 8**,** e73005.

SMYKAL, V., BAJGAR, A., PROVAZNIK, J., FEXOVA, S., BURICOVA, M., TAKAKI, K., HODKOVA, M., JINDRA, M. & DOLEZEL, D. 2014. Juvenile hormone signaling during reproduction and development of the linden bug, Pyrrhocoris apterus. *Insect biochemistry and molecular biology,* 45**,** 69-76.

SMYKAL, V. & RAIKHEL, A. S. 2015. Nutritional control of insect reproduction. *Current opinion in insect science,* 11**,** 31-38.

SONG, J., WU, Z., WANG, Z., DENG, S. & ZHOU, S. 2014. Krüppel-homolog 1 mediates juvenile hormone action to promote vitellogenesis and oocyte maturation in the migratory locust. *Insect biochemistry and molecular biology,* 52**,** 94-101.

SORGE, D., NAUEN, R., RANGE, S. & HOFFMANN, K. H. 2000. Regulation of vitellogenesis in the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *Journal of Insect Physiology,* 46**,** 969-976.

SPINDLER, K. & SPINDLER-BARTH, M. 1991. Ecdysteroid production and metabolism by an epithelial cell line from *Chironomus tentans*. *Naturwissenschaften,* 78**,** 78-79.

STAY, B. & TOBE, S. S. 2007. The role of allatostatins in juvenile hormone synthesis in insects and crustaceans. *Annu. Rev. Entomol.,* 52**,** 277-299.

SUN, C., HU, L., LIU, S., GAO, Z. & ZHANG, S. 2013. Functional analysis of domain of unknown function (DUF) 1943, DUF1944 and von Willebrand factor type D domain (VWD) in vitellogenin2 in zebrafish. *Dev Comp Immunol,* 41**,** 469-476.

SUN, Y., XIAO, L., CAO, G., ZHANG, Y., XIAO, Y., XU, G., ZHAO, J., TAN, Y. & BAI, L. 2016. Molecular characterisation of the vitellogenin gene (AlVg) and its

expression after *Apolygus lucorum* had fed on different hosts. *Pest management science*.

TAMBORINDEGUY, C., HUOT, O. B., IBANEZ, F. & LEVY, J. 2017. The influence of bacteria on multi-trophic interactions among plants, psyllids, and pathogen. *Insect Science*.

TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M. & KUMAR, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution,* 28**,** 2731-2739.

TERESANI, G. R., BERTOLINI, E., ALFARO-FERNÁNDEZ, A., MARTÍNEZ, C., TANAKA, F. A. O., KITAJIMA, E. W., ROSELLÓ, M., SANJUÁN, S., FERRÁNDIZ, J. C. & LÓPEZ, M. M. 2014. Association of 'Candidatus Liberibacter solanacearum'with a vegetative disorder of celery in Spain and development of a real-time PCR method for its detection. *Phytopathology,* 104**,** 804-811.

TEULON, D., WORKMAN, P., THOMAS, K. & NIELSEN, M. 2009. Bactericera cockerelli: incursion, dispersal and current distribution on vegetable crops in New Zealand. *New Zealand Plant Protection,* 62**,** 136-144.

THOMPSON, D. M., KHALIL, S. M., JEFFERS, L. A., SONENSHINE, D. E., MITCHELL, R. D., OSGOOD, C. J. & ROE, R. M. 2007. Sequence and the

developmental and tissue-specific regulation of the first complete vitellogenin messenger RNA from ticks responsible for heme sequestration. *Insect biochemistry and molecular biology,* 37**,** 363-374.

TSUCHIDA, K., NAGATA, M. & SUZUKI, A. 1987. Hormonal control of ovarian development in the silkworm, *Bombyx mori*. *Archives of insect biochemistry and physiology,* 5**,** 167-177.

TUFAIL, M., BEMBENEK, J., ELGENDY, A. M. & TAKEDA, M. 2007. Evidence for two vitellogenin-related genes in *Leucophaea maderae*: The protein primary structure and its processing. *Archives of insect biochemistry and physiology,* 66**,** 190-203.

TUFAIL, M., HATAKEYAMA, M. & TAKEDA, M. 2001. Molecular evidence for two vitellogenin genes and processing of vitellogenins in the American cockroach, *Periplaneta americana*. *Archives of insect biochemistry and physiology,* 48**,** 72-80.

TUFAIL, M., NAEEMULLAH, M., ELMOGY, M., SHARMA, P., TAKEDA, M. & NAKAMURA, C. 2010. Molecular cloning, transcriptional regulation, and differential expression profiling of vitellogenin in two wing-morphs of the brown planthopper, *Nilaparvata lugens* Stål (Hemiptera: Delphacidae). *Insect molecular biology,* 19**,** 787-798.

TUFAIL, M., NAGABA, Y., ELGENDY, A. M. & TAKEDA, M. 2014. Regulation of vitellogenin genes in insects. *Entomological Science,* 17**,** 269-282.

TUFAIL, M., RAIKHEL, A. S. & TAKEDA, M. 2005. Biosynthesis and processing of insect vitellogenins. *Progress in vitellogenesis. Reproductive biology of invertebrates,* 12**,** 1-32.

TUFAIL, M. & TAKEDA, M. 2002. Vitellogenin of the cockroach, *Leucophaea maderae*: nucleotide sequence, structure and analysis of processing in the fat body and oocytes. *Insect biochemistry and molecular biology,* 32**,** 1469-1476.

TUFAIL, M. & TAKEDA, M. 2008. Molecular characteristics of insect vitellogenins. *Journal of Insect Physiology,* 54**,** 1447-1458.

UNTERGASSER, A., CUTCUTACHE, I., KORESSAAR, T., YE, J., FAIRCLOTH, B. C., REMM, M. & ROZEN, S. G. 2012. Primer3-new capabilities and interfaces. *Nucleic acids research,* 40**,** e115-e115.

VAFOPOULOU, X. & STEEL, C. G. 2005. Testis ecdysiotropic peptides in *Rhodnius prolixus*: Biological activity and distribution in the nervous system and testis. *Journal of insect physiology,* 51**,** 1227-1239.

WALLIS, C. M., CHEN, J. C. & CIVEROLO, E. L. 2012. Zebra chip-diseased potato tubers are characterized by increased levels of host phenolics, amino acids, and defense-related proteins. *Physiological and Molecular Plant Pathology,* 78**,** 66-72.

WALLIS, C. M., RASHED, A., CHEN, J., PAETZOLD, L., WORKNEH, F. & RUSH, C. M. 2015. Effects of potato-psyllid-vectored 'Candidatus Liberibacter solanacearum' Infection on potato leaf and stem physiology. *Phytopathology,* 105**,** 189-198.

WALLIS, R. L. 1955. *Ecological studies on the potato psyllid as a pest of potatoes*, US Dept. of Agriculture.

WANG, X.-W., LUAN, J.-B., LI, J.-M., BAO, Y.-Y., ZHANG, C.-X. & LIU, S.-S. 2010. De novo characterization of a whitefly transcriptome and analysis of its gene expression during development. *BMC genomics,* 11**,** 400.

WEAVER, R. J. & EDWARDS, J. P. 1990. The role of the corpora allata and associated nerves in the regulation of ovarian cycles in the oviparous cockroach *Periplaneta americana*. *Journal of Insect Physiology,* 36**,** 51-59.

WHEELER, A. G. 2001. *Biology of the plant bugs (Hemiptera: Miridae): pests, predators, opportunists*, Cornell University Press.

WIGGLESWORTH, S. V. B. 1970. Insect hormones. *Insect hormones.*

WILLIAMS, D. 1996. A brief account of the hibiscus mealybug *Maconellicoccus hirsutus* (Hemiptera: Pseudococcidae), a pest of agriculture and horticulture, with descriptions of two related species from southern Asia. *Bulletin of Entomological Research,* 86**,** 617-628.

WU, L. T., HUI, J. H. & CHU, K. H. 2013. Origin and evolution of yolk proteins: expansion and functional diversification of large lipid transfer protein superfamily. *Biol Reprod,* 88**,** 102.

WYATT, G. R. & DAVEY, K. G. 1996. Cellular and molecular actions of juvenile hormone. II. Roles of juvenile hormone in adult insects. *In:* EVANS, P. D. (ed.) *Advances in Insect Physiology.* Academic Press.

YANG, X.-B. & LIU, T.-X. 2009. Life history and life tables of *Bactericera cockerelli* (Homoptera: Psyllidae) on eggplant and bell pepper. *Environmental entomology,* 38**,** 1661-1667.

YAO, J., SAENKHAM, P., LEVY, J., IBANEZ, F., NOROY, C., MENDOZA, A., HUOT, O., MEYER, D. F. & TAMBORINDEGUY, C. 2016. Interactions "Candidatus Liberibacter solanacearum"—Bactericera cockerelli: Haplotype Effect on Vector Fitness and Gene Expression Analyses. *Frontiers in Cellular and Infection Microbiology,* 6.

ZENG, B., HUANG, Y., XU, J., SHIOTSUKI, T., BAI, H., PALLI, S. R., HUANG, Y. & TAN, A. 2017. The FOXO transcription factor controls insect growth and development by regulating juvenile hormone degradation in the silkworm, Bombyx mori. *Journal of Biological Chemistry***,** jbc. M117. 777797.

ZHANG, S., WANG, S., LI, H. & LI, L. 2011. Vitellogenin, a multivalent sensor and an antimicrobial effector. *The international journal of biochemistry & cell biology,* 43**,** 303-305.

ZHU, J., MIURA, K., CHEN, L. & RAIKHEL, A. S. 2000. AHR38, a homolog of NGFI-B, inhibits formation of the functional ecdysteroid receptor in the mosquito Aedes aegypti. *The EMBO journal,* 19**,** 253-262.

ZHU, J., MIURA, K., CHEN, L. & RAIKHEL, A. S. 2003. Cyclicity of mosquito vitellogenic ecdysteroid-mediated signaling is modulated by alternative dimerization of the RXR homologue Ultraspiracle. *Proceedings of the National Academy of Sciences,* 100**,** 544-549.