ABSTRACT

CABRERA, ANA ROSA. Advances in Resistance Monitoring of Agricultural Pests and in the Elucidation of Mite Reproductive Physiology. (Under the direction of R. Michael Roe.)

The work conducted for this dissertation aimed to contribute to our knowledge regarding resistance monitoring of agricultural pests and mite reproduction. Resistance monitoring of lepidopteran pests exposed to transgenic cotton expressing *Bacillus thuringiensis* (Bt) toxins is necessary and required to prevent the development of insect resistance. A bioassay was developed using Bt cotton plant extracts to rehydrate a heliothine diet and observe feeding disruption of the cotton pest *Heliothis virescens*. A diagnostic dose was estimated for two different pyramided Bt cotton varieties. The bioassay was evaluated with field *H. virescens* populations from North Carolina and two Bt resistant, laboratory strains. Ready to use meal pads containing Bt from cotton plants can be stored for up to 5 months. This bioassay is practical, lower cost and can be adapted for other Bt cotton varieties and Bt crops. This work is described in chapter 1.

Mites are important medical and agricultural pests. Currently, there is limited information regarding the regulation of female reproduction in mites and few studies have examined mite yolk proteins. A review of the literature was conducted regarding the regulation of female reproduction in mites and a new model for the regulation of vitellogenesis in the Acari was proposed. Relevant work on the regulation of vitellogenesis in insects, crustaceans and ticks, as well as observations on the effects of some insect hormones

and their analogs on mite reproduction, lead us to conclude that the prevailing assumption that mites regulate vitellogenesis with high levels of juvenile hormone (JH) may not be correct. As a result of this review a new unifying model for the Acari was developed where ecdysteroids, and not JH, regulate vitellogenesis in mites. This review was published in 2009 in the Journal of Insect Physiology and is presented in chapter 2.

In chapter 3, the characterization of the major yolk protein vitellin of the twospotted spider mite, *Tetranychus urticae*, is presented. This work determined that *T. urticae* vitellin is a glycolipoprotein, although the carbohydrate and lipid content appears to be lower than that of the American dog tick, *Dermacentor variabilis*. It was clear that spider mite vitellin does not carry heme, a fundamental difference with the tick yolk protein. *T. urticae* vitellin migrated as a single band under native-PAGE conditions, but five different bands were observed with isoelectric focusing analysis, indicating that multiple Vgs may be expressed. This conclusion is also supported by the recent evidence that *T. urticae* ovipositing females express at least 4 vitellogenin (Vg) genes. This work was published in the Journal of Insect Physiology in 2009.

Finally, the transcriptome analysis of the predatory mite, *Phytoseiulus persimilis*, is presented in chapter 4. This is the first transcriptome analysis of a mite and is a result of a 454 pyrosequencing project that yielded 12,556 sequences of transcripts. From those, 11 contigs were similar to arthropod Vgs and 6 to Vg-receptors (VgR). These contigs were further studied with available tools such as the open reading frame finder in Genbank, and alignments with ClustalW and stage-specific expression studies were conducted with selected

Vg (6) and VgR (3) contigs. Two Vg contigs (11791 and 12365) are likely to correspond to two different Vg genes. Obtaining DNA-sequence information of mite Vg and VgR genes will facilitate the study of the regulation of female reproduction in mites, which may lead to new ways of mite pest control.

Advances in Resistance Monitoring of Agricultural Pests and in the Elucidation of Mite Reproductive Physiology

by
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DEDICATION

This dissertation is dedicated to my mom and dad, Amanda Josefina Cordón Orellana de Cabrera and Javier Humberto Cabrera Sagastume. Thank you for your love and support. I know sometimes you don't understand me, but you always let me be myself.

Esta disertación esta dedicada a mi mama y mi papa, Amanda Josefina Cordón Orellana de Cabrera and Javier Humberto Cabrera Sagastume. Gracias por su amor y apoyo. Sé que a veces no me entienden, pero siempre me permiten ser yo misma.

BIOGRAPHY

Ana Rosa Cabrera was born and raised in Chiquimula, Guatemala. At 17 yrs old she left her home and country to study in the Pan-American School of Agriculture, El Zamorano, in Honduras. During her undergrad program at Zamorano, she quickly became interested in agricultural pest management. She regards this time as one of the happiest in her life.

After a brief internship in Connecticut where she learned to speak English at the age of 21, she knew she wanted to continue her education at the graduate level. In 2001, she entered the M. Sc. program in the Department of Natural Resources and Environmental Sciences at the University of Illinois, Urbana-Champaign. At some point during this period she decided she wanted to become an entomologist.

Ana started her Ph. D. program in Entomology at North Carolina State University during the Fall 2006. She joined the laboratory of Dr. Michael Roe where she discovered that insect physiology is a cool area of research and that she loves to learn about it. She will officially be an entomologist for the rest of her life and that thought makes her smile. Ana doesn't know what life will bring her, but she has the feeling that from now on, everything will be alright.

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Dr. Christine Casey was critical for my application and acceptance in the Department of Entomology at NCSU and I'd like to thank her for that. The financial support during my program came through the Department of Entomology of NCSU (teaching assistantship) and Cotton Inc. (research assistantship). In addition, I'd like to thank my professors at NCSU for teaching me about insects, molecular biology and other cool stuff.

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Chapter 1

A bioassay used as an artificial cotton leaf for resistance monitoring of tobacco budworm, *Heliothis virescens* (Lepidoptera: Noctuidae), to pyramided cotton varieties

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Abstract

The development of insect resistance to genetically modified crops expressing *Bacillus thuringiensis* (Bt) toxins is of concern because of the potential economic and environmental impacts. Thus far, reports show that the cotton pest known as tobacco budworm, *Heliothis virescens*, is susceptible to the commercially available Bt cotton varieties in the United States. However, resistance monitoring is necessary to prevent or delay the development of resistance, and is also mandated by the Environmental Protection Agency. Current monitoring techniques take time and can be expensive. Therefore, the objective of this project was to develop a practical, reliable bioassay, to be used as an artificial cotton leaf as a resistance monitoring tool for *H. virescens* that is relevant to new, pyramided Bt cotton varieties. The bioassay was based on a previously described feeding disruption test (FDT) from our laboratory. Cotton leaves were used as the source of Bt toxins, added to a hydratable, artificial diet containing a blue indicator. Using neonate larvae from a Bt-susceptible, laboratory reared *H. virescens* strain, we determined a diagnostic dose for Bollgard II (1.3 µg of cotton / meal pad) and Widestrike cotton (42.5 µg of cotton / meal pad) that prevented the production of blue feces. At the diagnostic dose, Bt-susceptible *H. virescens* larvae produced 0-2 fecal pellets after 24 hrs, while Bt-resistant larvae produced >2 fecal pellets. The artificial cotton leaves were evaluated with three different field populations of tobacco budworm from central North Carolina. The diagnostic doses were also evaluated with two different Bt-resistant, laboratory reared *H. virescens* strains: YHD2 (resistant to Cry1Ac) and CxC (resistant to Cry2A). The ready-to-use FDTs can be stored up to 5 months

at room temperature. The application of the artificial leaf bioassay as an easy to use monitoring tool is discussed.

1. Introduction

The cultivation of transgenic crops expressing *Bacillus thuringiensis* (Bt) toxins to control insect pests has shown to provide economical (Shelton et al., 2002) and environmental benefits due to the reduction in the use of chemical insecticides (Ferré and Van Rie, 2002), while their impact on arthropod composition in the field, including non-target organisms, is minimal (Sisterson et al., 2004). Since their introduction in the 1990s, the proportion of US acreage planted with Bt-cotton cultivars has grown steadily (May et al., 2003). Yet, one of the major concerns with the use of Bt crops is the development of resistance in the target insect pest populations due to their constant exposure to the toxins through the transgenic plants (Ferré and Van Rie, 2002; Gould, 1998).

Resistance mechanisms to Bt toxins have been studied and characterized in some insects. These mechanisms include a reduction of proteolytic activity in the insect gut, Bt receptor modifications, and midgut cell regeneration or replacement (Ferré and Van Rie, 2002). Prior to the commercialization of "pyramided" cultivars that express two or more Bt toxins, Gould (1998) had already suggested that this strategy would reduce the risk of development of insect resistance in the field, because some field observations indicated the initial resistance allele frequency to be low, and other studies suggested that Bt resistance is a

recessive or partially recessive trait. Zaho et al. (2003) showed experimentally that Bt pyramided broccoli delayed the evolution of resistance in the diamondback moth, *Plutella xylostella*, compared to broccoli expressing a single Bt toxin. Pyramided cotton varieties reached the market during the early 2000s, not only as a response to resistance management, but also to improve the inconsistent control that one-toxin cultivars provided against some lepidopteran pests such as the bollworm, *Helicoverpa zea* (Jackson et al., 2007). Because changes in Bt binding sites in the insect gut seem to be the most common of the mechanisms of resistance, the pyramided cultivars that have been developed express different Bt-toxins that bind to different receptors (Ferré and Van Rie, 2002).

Although the likelihood of resistance development is lowered by the implementation of the pyramiding strategy, there are several examples of insect resistance to multiple chemical insecticides, and a resistance management approach should be implemented (Shelton et al., 2002). Selection for resistance to Bt toxins has been demonstrated under laboratory conditions (Gould et al., 1997); one study showed that *H. virescens* developed resistance to Cry1Ac to levels of >10,000 fold compared to susceptible strains, and cases of cross resistance from Cry1Ac to Cry1Ab and Cry1Fa have been observed (Ferré and Van Rie, 2002). Implemented resistance management strategies have included utilizing high dosages and refugia in the field (Gould, 1998); this integrated system should be accompanied by a monitoring methodology to help detect changes in resistance allele frequencies at early stages to guide making appropriate management decisions (Shelton et al., 2002).

Monitoring bioassays based on diagnostic doses of Bt toxins could be useful tools to consistently screen lepidopteran pest populations in Bt cotton (Blanco et al., 2008). Previous work by Bailey et al. (1998, 2001) showed that a feeding disruption bioassay with Cry1Ac toxin incorporated into an artificial diet containing blue dye could be used to monitor tobacco budworm resistance development by quantifying the production of blue fecal pellets. The same bioassay could also be used for species diagnosis, to identify *H. virescens* and *H. zea* larvae collected in the field (Bailey 1998, 2001). Roe et al. (2000, 2003) introduced a novel plate (containing dry meal pads of the artificial diet) with a white background to facilitate observation of blue feces production. With this new technology, diagnostic doses for tobacco budworm and bollworm were estimated for Cry1Ac, Cry1Ab and Cry1F by rehydrating the diet with Bt toxins in water (Roe et al. 2005). Monitoring bioassays are mainly based on exposure of the target insect to diet containing a Bt toxin obtained from transformed bacteria, such as *Pseudomonas* spp. (Bailey 1998, 2001; Blanco et al., 2008). A disadvantage of using this source includes the limited availability of Bt toxins due to their high cost of production and purification. At a large scale, this can increase the expense of the monitoring technique. One way to reduce cost could be to add the toxin in a suspension on top of the artificial diet instead of incorporating the toxin into the diet, which typically would require about 2 times more toxin to obtain similar results (Blanco et al., 2008). Another way of reducing costs would be using other sources of Bt toxins, such as transgenic plants. Thus, the objective of the present study was to develop an "artificial leaf bioassay" based on the exposure of

neonate larvae from *H. virescens* to an artificial diet rehydrated with pyramided Bt cotton leaf extracts as the source of Bt toxins.

2. Materials and Methods

2.1 Leaf extracts preparation and bioassay description

Leaves from 8-week old cotton plants were used to prepare leaf extracts, used as the source of *Bacillus thuringiensis* (Bt) toxins. Cotton varieties used in this study were: 1) Non-Bt cotton PHY 425 RF (Dow AgroSciences, Indianapolis, IN), 2) Bollgard II cotton DP 161 B2RF, expressing *Bacillus thuringiensis* Cry1Ac and Cry2Ab toxins (Monsanto, St. Louis, MO), and 3) Widestrike cotton PHY 485 WRF, expressing *Bacillus thuringiensis* Cry1Ac and Cry1F toxins (Dow AgroSciences, Indianapolis, IN). The plants were grown under greenhouse conditions, with temperature ranging from 22-31 ºC and natural light conditions throughout the year. After harvest the leaves were kept at -80 ºC until used.

A leaf extract stock solution was prepared for each cotton variety using 4-ml of distilled water for each gram of leaf tissue. The leaf tissue was homogenized using a Polytron homogenizer (PCU, Kinematica, Switzerland) for ~5min and then the preparation was filtrated using glass wool. The resulting leaf extract solution was expressed as 0.25 µg of cotton per µl (a relative measurement); the different cotton solutions were kept at -80 ºC until used.

We used plastic plates containing 16 wells as previously designed and described for feeding disruption tests (FDT) (Roe et al. 2005). The wells contained 200 µl of a freeze dried heliothine artificial diet (Burton, 1970). The diet was rehydrated with 170 µl of leaf extract and allowed to absorb moisture for approximately 2 hrs. Then one 0-24 hr old larva of tobacco budworm was placed in each well. The plates were covered with an adhesive plastic cover that allowed the exchange of air into the well but restricted the larvae to the well (Figure 1). After 24 hrs, the number of fecal pellets was counted for each larva.

2.2 Determining a diagnostic dose

The bioassay described in the previous section was utilized to determine a diagnostic dose for Bollgard II and Widestrike. We evaluated 0.7, 1.3, 2.6 and 5.3 µg of Bollgard II cotton per meal pad and 5.3, 10.6, 21.2 and 42.5 µg of Widestrike cotton per meal pad. The leaf extract stock solutions were expressed as 0.25 µg of cotton per µl (see previous section). For each cotton variety, an undiluted Non-Bt cotton treatment was included as a control (42.5 µg / meal pad). For each treatment (dose), four 16-well plates were used for a total of 64 larvae. The diagnostic dose was determined as the dose that resulted in 0-2 fecal pellets per larvae after 24 hrs, for all the larvae tested (see results). The tobacco budworm larvae used in these experiments were from a Bt-susceptible, laboratory reared strain Hv02 (NCSU, Raleigh, NC).

2.3 Field validation of diagnostic doses with H. virescens collected from tobacco fields

The diagnostic doses for the larval FDT were also evaluated with field populations from three different sites in North Carolina. Tobacco budworm eggs were collected from tobacco fields near the town of Cameron in Moore Co (August 25, 2008), Clayton in Johnston Co. (June 30, 2009), and Reidsville in Rockinham Co. (July 20, 2009). Tobacco buds with one or more eggs were brought back to the laboratory and most of the plant tissue was removed to prevent larval feeding on plant material after eclosion. The eggs were placed in a Styrofoam container at 27 °C and 65% relative humidity. Newly hatched larvae (0-24 hr old) were used for the evaluation of the diagnostic doses with bioassays, as described above. The sample size for Bollgard II and the corresponding Non Bt control was 60 and 61 from Moore Co, 26 and 30 from Johnston Co., and 52 and 44 from Rockingham Co. Likewise, the sample size from Widestrike and its Non Bt control was 60 and 62 from Moore Co., 32 and 25 from Johnston Co., and 51 and 51 from Rockingham Co.

2.4 Evaluation of the diagnostic dose with two different H. virescens Bt-resistant strains

Once the diagnostic doses were established for Bollgard II (1.3 µg / meal pad) and Widestrike (42.5 µg / meal pad) cotton, they were evaluated with two different Bt-resistant, laboratory reared *H. virescens* strains. These strains were reared in the laboratory of Dr. Fred Gould (NCSU, Raleigh, NC), who provided us with larvae for these bioassays. Strain YHD2 had been selected for resistance to Cry1Ac, although cross-resistance to Cry1A, Cry1Fa and

Cry2A had been observed (Jackson et al., 2007). Strain CxC had been selected for resistance to Cry1A and Cry2Aa2 (Jackson et al., 2007). The bioassays were conducted as previously described with four 16-well plates used for each treatment, for a total of 64 larvae per treatment. A control treatment with Non-Bt cotton was included for each of the cotton varieties (1.3 and 42.5 µg of cotton / meal pad).

2.5 FDT storage

The shelf life of ready-to-use FDTs was evaluated over a period of six months. Meal pads were rehydrated with either Non-Bt or Bollgard II cotton leaf extracts (1.3 µg cotton / meal pad). Afterward, the diet was lyophilized at -50 °C during 24 hrs. Plates were packed in plastic bags with a desiccant followed by air removal using a vacuum food sealer (Rival® Seal a Meal, The Holmes Group, Inc., Milford, MA), and then stored at room temperature. The diet was rehydrated with 150 µl of distilled water and allowed to rehydrate for approximately 1 hr. Then, the bioassay was conducted as described before, with susceptible *H. virescens* strain Hv02. The plates were evaluated each month, and four 16-well plates were used for each treatment for a total of 64 larvae.

2.6 Statistics

We estimated 95% confidence intervals for the evaluation of the diagnostic dose with field populations of *H. virescens,* and the comparisons between Bollgard II and Widestrike cotton with their respective controls. Comparisons between each cotton variety (Bollgard II and Widestrike) and their corresponding control (Non-Bt) were conducted with t-tests for the validation of a diagnostic dose using resistant, laboratory reared *H. virescens* strains YHD2 and CxC. The data set of strain CxC for the comparison of Non-Bt and Widestrike cotton was transformed with square root, to attain equal variances between treatments. Finally, for the evaluation of the ready-to-use FDTs shelf life, we used t-tests for each pair-wise comparison (Non-Bt vs. Bollgard II) for each month. We set our Type I error rate at 0.05 for each of the different, independent statistical tests. The program SAS version 9.1.3 (SAS Institute, 2003) was used to conduct the statistical analysis.

3. Results and Discussion

3.1 The diagnostic doses

The diagnostic dose for the artificial leaf assay based on feeding disruption for susceptible, laboratory reared *Heliothis virescens* larvae with Bollgard II cotton, was determined as the lowest dose that produced 0-2 fecal pellets per larva, in all tested larvae. We estimated the Bollgard II cotton diagnostic dose at 1.3 μg per meal pad (Figures 1, 2). In

contrast, all larvae exposed to meal pads rehydrated with Non-Bt cotton produced >2 fecal pellets (mean= 28.5 ± 1.3, range= 9 - 79 fecal pellets) after 24 hrs.

Likewise, the diagnostic dose for Widestrike cotton was estimated at 42.5 µg per meal pad (Figure 2). This dose produced 0-2 fecal pellets in all tested larvae. Tobacco budworm neonates exposed to meal pads rehydrated with Non-Bt cotton produced >2 fecal pellets (mean= 14.2 ± 2.7, range= 0 - 46 fecal pellets) after 24 hrs. In this case, 1.6% of the larvae produced 0-2 fecal pellets (false negatives). Figure 1 depicts neonate *H. virescens* larvae after 24 hrs on wells containing meal pads rehydrated with Bollgard II and Widestrike cotton, and their respective controls.

The diagnostic dose for Bollgard II cotton (1.3 µg / meal pad) was 32.7 x lower than the diagnostic dose determined for Widestrike II cotton (42.5 µg / meal pad). The observed difference in the amount of fresh plant tissue required to stop fecal production between these varieties may be due to the type of Bt-Cry toxins expressed, the levels of expression of those toxins on an 8-week old cotton plant, or both. Previous work on bioassays based on a diagnostic dose on artificial diet have used single Bt toxins. This is the first effort to develop a bioassay based on the same principles using Bt cotton plants as the source of Bt toxin, with two Bt toxins expressed by the pyramided varieties used (Bollgard II and Widestrike).

Baseline susceptibility estimates of *H. virescens* to Bt toxins based on diagnostic doses of Bt toxins can vary greatly between laboratory colonies, field populations, host plant

and time of the year of collections (Alli and Luttrell, 2007). Most of these estimates have been based on the evaluation of mortality caused by the Bt toxins (LC_{50}). Some examples of diagnostic doses for *H. virescens* to Bt toxins include 100 ng Cry1F /cm2 (Blanco et al., 2008), 1.6 µg Cry2Ab2/ml of diet (Alli and Luttrell, 2007). There are several factors that can affect the determination of a diagnostic dose, for example, Gore et al. (2005) observed that neonate larvae from tobacco budworm and bollworm avoided feeding on an artificial diet containing either Cry1Ac or Cry2Ab. Avoidance of feeding on Bt-cotton has also been observed in another lepidopteran species, *Trichoplusia ni*, considered a secondary pest of cotton (Li et al., 2006). The estimates also vary depending on whether the toxin is incorporated into the diet or is applied on top of it (Blanco et al., 2008). Thus, direct comparison of our results with other studies is difficult.

3.2 An artificial cotton leaf to monitor resistance in tobacco budworm field populations

The diagnostic doses for Bollgard II and Widestrike cotton were evaluated to monitor Bt susceptibility in three different tobacco budworm field populations from the central region of North Carolina. Tobacco budworm larvae collected from the field and exposed to Bollgard II treated meal pads did not produce any fecal pellets after 24 hr (Cameron, Moore Co., mean= 0, n= 60; Clayton, Johnston Co., mean= 0, n= 26; Reidsville, Rockinham Co., mean= 0, n= 52) (Figure 3). In contrast, larvae that fed on the corresponding Non-Bt cotton treated meal pads produced >2 fecal pellets (Cameron, Moore Co., mean= 10.1, n= 61;

Clayton, Johnston Co., mean= 24.7, n= 30; Reidsville, Rockinham Co., mean= 17.9, n=44). However, a number of non-feeding larvae (false negatives) were observed in the Non-Bt cotton treatment (3.3% of larvae collected from Cameron and 2.2% of the larvae collected from Reidsville).

Similar results were observed with tobacco budworm larvae from field collected eggs feeding on Widestrike treated meal pads. All the tested larvae produced 0-2 fecal pellets at 24 hrs on the Widestrike-treated pads (Cameron, Moore Co., mean= 0.1, n= 60; Clayton, Johnston Co., mean= 0.1, n= 32**;** Reidsville, Rockinham Co., mean= 0, n= 51). The corresponding Non-Bt treatment yielded the following results of number of fecal pellets for larvae collected from 1) Cameron, mean= 11.2 (n= 62), 2) Clayton, mean= 17.2 (n= 25) and Reidsville, mean= 17.9 (n= 51) (Figure 3). In this case, the non-feeding larvae represented 9.5%, 4% and 6.8% of tested larvae for Cameron, Clayton and Reidsville, respectively.

These field collections for the validation of the diagnostic doses of Bollgard II and Widestrike showed that this bioassay can be used for diagnosis of Bt susceptibility in *H. virescens* field populations. All the larvae exposed to diet containing Bt cotton produced 0-2 fecal pellets, while most of the larvae feeding on Non-Bt cotton treated diet produced >2 fecal pellets (Figure 3). Our results show that the tobacco budworm populations sampled in Moore, Johnston and Rockinham counties in North Carolina are susceptible to Bollgard II and Widestrike cotton. To date, there are no reported cases of field development of insect resistance to Bt crops (Bates et al., 2005).

On average, field collected tobacco budworm larvae produced fewer fecal pellets and a larger proportion were non-feeders compared to the laboratory reared susceptible strain. Similar observations of non-feeding larvae exposed to Non-Bt treatments were made by Bailey et al. (2001) when evaluating a Cry1Ac diagnostic dose with field collected tobacco budworm populations from Louisiana, North Carolina, Georgia and Mississippi. These results may suggest an adaptation to feeding on an artificial diet in the laboratory strain. Tobacco budworm populations vary in the level of susceptibility to Bt toxins. For example, Alli et al. (2006) estimated LC_{50}s for Cry1Ac for several *H. virescens* strains, including laboratory colonies and field populations. Combined estimation of LD_{50} for laboratory colonies was almost 5-fold lower than the combined LC_{50} estimation for field populations (Alli et al., 2006). Variation in Bt toxin susceptibility has also been observed between tobacco budworm populations collected in different crops. A study by Alli and Luttrell (2007) showed that *H. virescens* larvae collected from tobacco fields had a higher LC50 than larvae collected from other crops including cotton and corn. Tobacco budworm populations sampled in our study were collected from tobacco fields; thus, it may be expected that these field strains would have different susceptibility than populations from other crops or the laboratory strain to the Bt toxins tested. Yet, the diagnostic doses selected for resistance monitoring for Bollgard II and Widestrike cotton showed that *H. virescens* from tobacco fields in central North Carolina are susceptible to these varieties.

3.3 Responses of Bt resistant H. virescens strains

Two different Bt-resistant, laboratory reared, *H. virescens* strains were used for the evaluation of the diagnostic doses for Bollgard II and Widestrike cotton. Newly hatched (0-24 hrs) larvae from the strain YHD2, resistant to the Bt toxin Cry1Ac, were fed on meal pads rehydrated with Bollgard II and Widestrike leaf extracts (Figure 4). Resistant YHD2 larvae produced >2 fecal pellets when exposed to meal pads containing Bollgard II at the diagnostic dose, 1.3 µg of cotton per meal pad. There were significant differences in the number of fecal pellets between larvae that feed on meal pads treated with Bollgard II (mean = 12.4) and Non-Bt cotton (mean= 30.1) ($t=7.69$, $P< 0.0001$) (Figure 4). However, 21.9% of larvae exposed to Bollgard II cotton produced 0-2 fecal pellets. When YHD2 larvae fed on Widestrike cotton, at the diagnostic dose 42.5 µg of cotton per meal pad, all the larvae produced >2 fecal pellets. There were no significant differences in the number of fecal pellets between YHD2 larvae that feed on meal pads treated with Widestrike (mean = 21.5) and Non-Bt cotton (mean = 23.5) ($t= 0.76$, $P=0.4476$).

Similarly, 0-24 hrs larvae from the strain CxC, resistant to the Bt-endotoxins Cry1Ac and Cry2A, fed on meal pads rehydrated with Bollgard II and Widestrike leaf extracts (Figure 5). Resistant CxC larvae produced >2 fecal pellets when exposed to meal pads containing Bollgard II at the diagnostic dose of 1.3 µg of cotton per meal pad. There were significant differences in the number of fecal pellets between larvae that fed on meal pads treated with Bollgard II (mean = 13.9) and Non-Bt cotton (mean= 31.7) ($t=6.49$, $P<0.0001$).

The occurrence of non-feeder larvae when fed on Bollgard II was 9.7%. When CxC larvae fed on Widestrike cotton, at the diagnostic dose of 42.5 µg of cotton per meal pad, approximately half of the larvae produced >2 fecal pellets, but 48.4% of the larvae were non-feeders. There were also significant differences in the number of fecal pellets between CxC larvae that fed on meal pads treated with Widestrike (mean = 17.8) and Non-Bt cotton (mean = 5.6) ($t= 8.46$, $P<0.001$).

Validation of the diagnostic dose with Bt resistant strains had some limitations. For example, non-feeder larvae were 21.9 and 9.7% for YHD2 and CxC strains, respectively, when exposed to diet containing Bollgard II cotton. Bollgard II cotton expresses Cry1Ac and Cry2Ab toxins, and strain YHD2 has been selected for resistance to Cry1Ac only, while strain CxC was originally resistant Cry1Ac and then selected for resistance to Cry2A (Jackson et al., 2007). Although cases of cross-resistance have been observed between some Bt toxins, i.e. *H. virescens* resistant to Cry1Ac showed some level of resistance to Cry1Ab and Cry1Fa (Ferré and Van Rie, 2002), the higher proportion of non-feeders observed for strain YHD2 may be due to the selection of this strain to one Bt toxin (Cry1Ac), while strain CxC has been exposed to two Bt toxins (Cry1Ac and Cry2A). These results may also be due to the levels of the different Bt toxins that these larvae were exposed to the Bt cotton varieties and to the type and number of receptors in the insect gut. Non-feeder larvae were not observed on experiments with strain YHD2 when exposed to Widestrike cotton, although YHD2 has not been exposed to Cry1F previously. Again, these results may be due to some level of cross-resistance between Cry1Ac and Cry1Fa (Ferré and Van Rie, 2002). On the

contrary, the high levels of non-feeder larvae were observed on strain CxC exposed to Widestrike (48.4%) may be an indication of the lack of cross-resistance between Cry2A and Cry1F. Additionally, Shaver et al. (1978) observed that gossypol -a polyphenol compound produced by cotton plants- and other compounds found in cotton flower bud extracts can be detrimental to tobacco budworm larvae, particularly neonates; these compounds may also have an effect of the feeding response of the tested larvae. Under laboratory conditions, tobacco budworm strains selected for resistance to a particular Bt toxin have shown cross-resistance to other Bt toxins to which the insects have not been exposed before. For example, the *H. virescens* strain CP73, selected for resistance to Cry1Ac was also resistant to Cry2Aa (Gahan et al. 2005). The nature of the resistance mechanisms for the same Bt toxin can be different between strains of the same insect species. As pointed out by Gahan et al. (2005), the *H. virescens* strains YHD2 and CP73 differ in the gene that allows resistance to Cry1Ac, which also demonstrates that resistance to a single Bt toxin can be conditioned by more than one single gene.

3.4 FDT storage

We evaluated the shelf life of ready-to-use FDTs, containing cotton leaf extracts from either Bollgard II or Non-Bt cotton for a period of six months. Our results suggested that the freeze-dried FDTs containing cotton tissue and stored at room temperature were viable for up to 5 months (Table 1). All susceptible, laboratory reared *H. virescens* larvae produced <2

fecal pellets when exposed to meal pads containing Bollgard II cotton (1.3 µg cotton / meal pad) stored for 1 – 5 months at room temperature. Most of the larvae that fed on meal pads with Bollgard II cotton that had been stored for 6 months also produced <2 fecal pellets, but the false positives accounted for 4.6% at this storage timing. The larvae exposed to meal pads with Non-Bt cotton fed on the diet and produced >2 fecal pellets even after 6 months of storage. The percentage of false negatives was 20.9% and 3.1% for months 1 and 2 respectively, and 0% for months 3 to 6.

We observed a trend, towards increasing number of fecal pellets produced by larvae exposed to Non-Bt treated diet as time of storage increased, which it is difficult to explain. Larvae used for this experiment were from different cohorts from the same susceptible, laboratory reared *H. virescens* colony (Hv02, NCSU). The feeding response of larvae from different cohorts might have differed in our experiment. This was also apparent in the number of non-feeder larvae exposed to Non-Bt treated meal pads, which accounted for 20.9% and 3.1% at months 1 and 2, respectively.

Overall, our results indicate that the artificial cotton leaf bioassay developed for monitoring tobacco budworm resistance to pyramided Bt cotton varieties is a useful, reliable technique. We developed the bioassay particularly for the pyramided varieties Bollgard II and Widestrike, but it could be also developed for other commercially available cotton varieties expressing one or more Bt toxins. An advantage of this bioassay technique is the availability of the source of Bt toxins obtained from plant extracts; these extracts could be

prepared in advance and then frozen for storage. The technique provides a practical, useful as a ready-to-use diagnostic tool with which resistance is determined by visual presence of fecal pellets.

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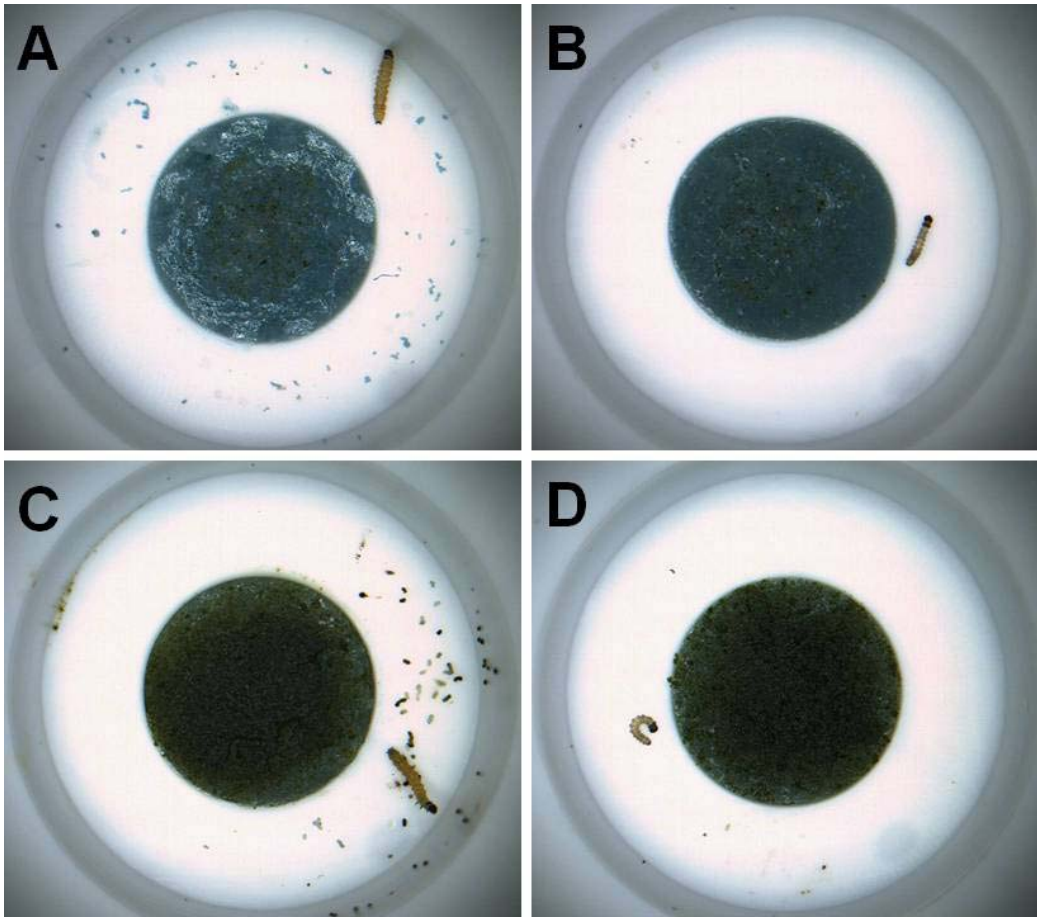

Figure 1. Individual wells, with meal pads rehydrated with leaf extract from A) Non-Bt cotton (1.3 µg / meal pad), B) Bollgard II (1.3 µg / meal pad), C) Non-Bt cotton (42.5 µg / meal pad) and D) Widestrike (42.5 µg / meal pad). Fecal pellets produced by neonate susceptible, laboratory reared *Heliothis virescens* are observed in wells with Non-Bt cotton treatments (A and C), while no fecal pellets are observed on Bt cotton treatments (B and D).

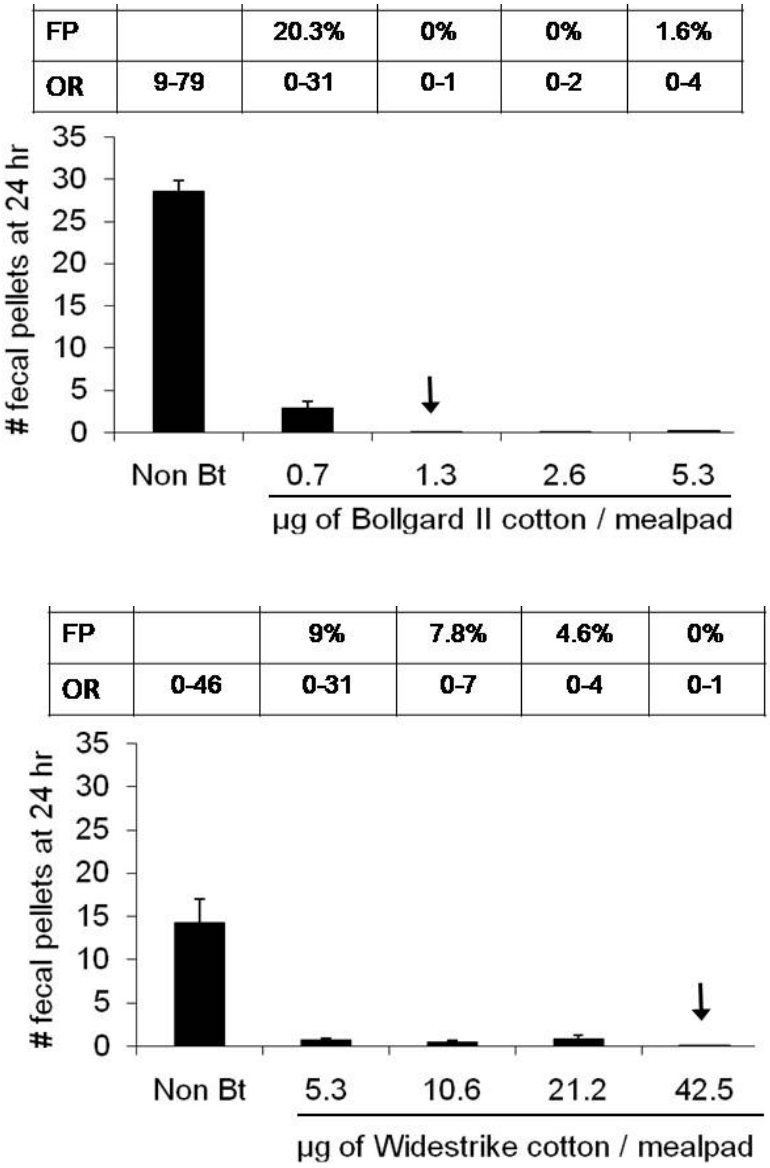

Figure 2. Mean number of fecal pellets (± 1SE) produced by susceptible, laboratory reared *Heliothis virescens* larvae, after 24 hr of exposure to Non Bt and different concentrations of Bt cotton treated meal pads. The arrow points to the concentration of Bollgard II (top) or Widestrike (bottom) cotton, selected as diagnostic dose. FP: false positives. OR: observed range (of fecal pellets).

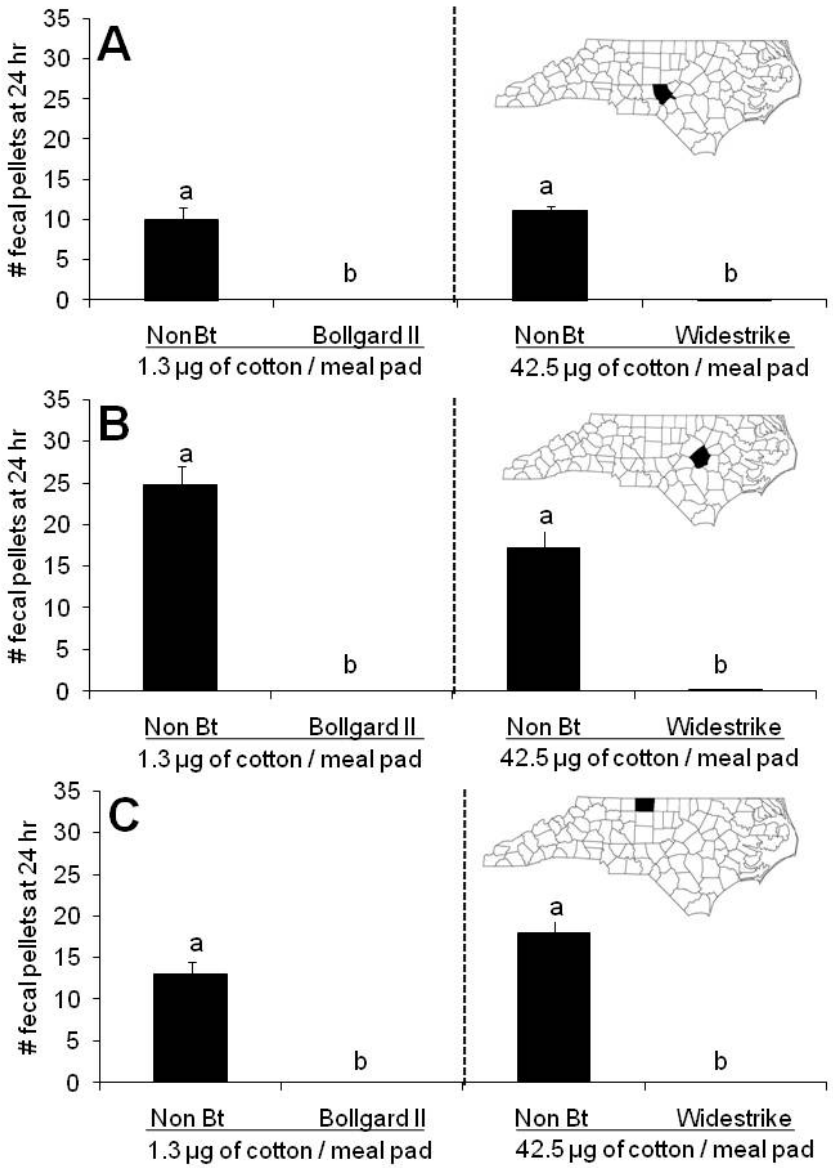

Figure 3. Mean number of fecal pellets (± SE) produced by field collected, neonate *Heliothis virescens* larvae feeding on meal pads treated with Non Bt or Bt cotton after 24 hr. Field populations were sampled from tobacco fields in Cameron, Moore Co. (A), Clayton, Johnston Co. (B) and Reidsville, Rockinham Co. (C), North Carolina. Bars with different letters represent significant statistical differences, $P \leq 0.05$.

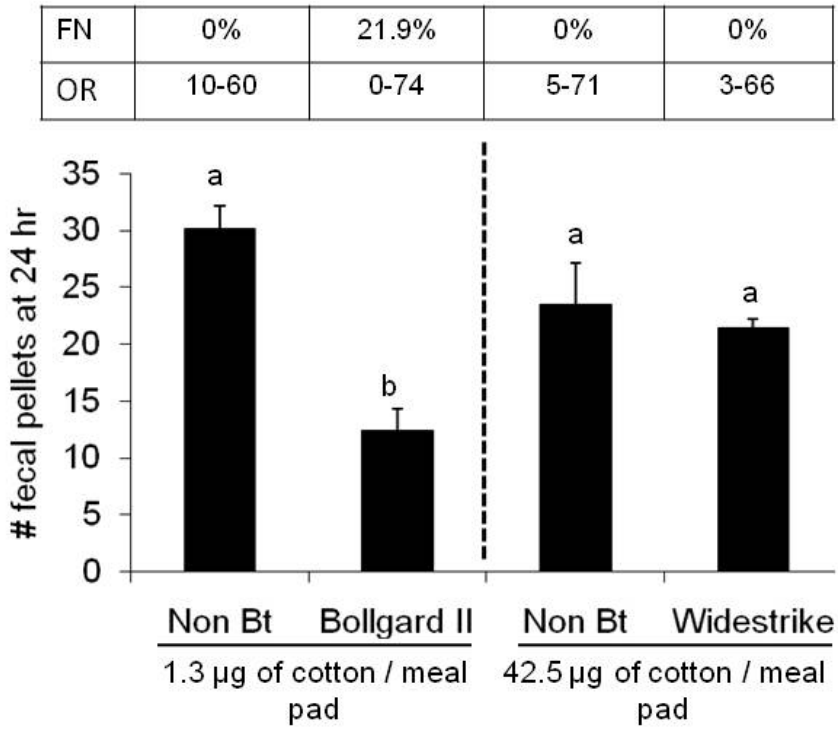

Figure 4. Mean number of fecal pellets (± 1SE) produced by resistant, laboratory reared *Heliothis virescens* larvae, strain YHD2, after 24 hr of exposure to Non Bt and Bt cotton treated meal pads. Bars with different letters represent significant statistical differences, P≤ 0.05. FN: false negatives. OR: observed range (of fecal pellets).

FN	0%	9.7%	4.7%	48.4%
OR	7-78	0-62	0-66	0-45

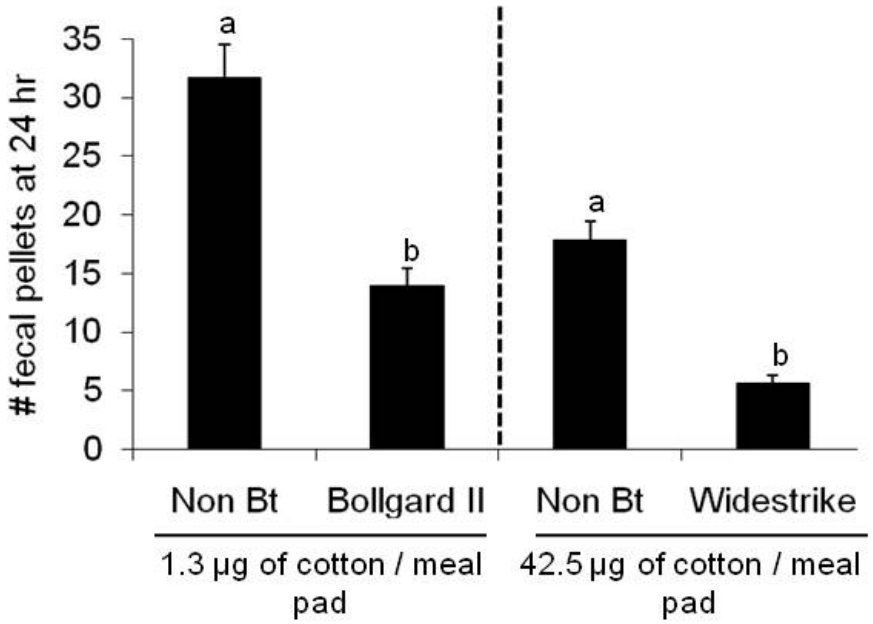

Figure 5. Mean number of fecal pellets (± 1SE) produced by resistant, laboratory reared *Heliothis virescens* larvae, strain CxC, after 24 hr of exposure to Non Bt and Bt cotton treated meal pads. Bars with different letters represent significant statistical differences, P≤ 0.05. FN: false negatives. OR: observed range (of fecal pellets).

Table 1. Evaluation of shelf life of ready-to-use plates containing meal pads with Non Bt and Bollgard II cotton during 6 months, stored at room temperature. Values represent mean number of fecal pellets (± 1SE) produced by susceptible, laboratory reared *Heliothis virescens* neonate larvae after 24 hr.

	Cotton variety		
Month	**Non Bt**	**Bollgard II**	**% false positives***
1	14.2 ± 1.5a	0.1 ± 0.03b	0
2	23.5 ± 2.05a	0.2 ± 0.06b	0
3	20.1 ± 1.62a	0.2 ± 0.07b	0
4	38.7 ± 2.53a	0.3 ± 0.08b	0
5	35.5 ± 1.60a	0.1 ± 0.10b	0
6	35.1 ± 1.96a	0.5 ± 0.16b	4.6

* false positive: susceptible larvae producing > 2 fecal pellets after 24 hrs, at the diagnostic dose

Chapter 2

Review

Regulation of female reproduction in mites: A unifying model for the Acari

This chapter was published in the *Journal of Insect Physiology* (2009; vol. 55, pp. 1079-1090) with the coauthors Kevin V. Donohue* and R. Michael Roe*.

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Abstract

It is well established in the literature that circulating high levels of juvenile hormone (JH) are responsible for the initiation of vitellogenesis and female reproduction in most insects studied so far. Exceptions include some Diptera, Lepidoptera and Hymenoptera. The current view is that JH also regulates yolk protein (vitellogenin, Vg) synthesis and female reproduction in mites. However, there is no published evidence that mites have the common insect JHs at any stage of their development. Also, research on the effects of exogenous applications of JH and JH analogs on the reproduction of mites is contradictory. Significant information is available on the life history of mite reproduction, and new information has become available on mite storage proteins including Vg. Although initial studies suggested that ticks may respond to exogenously applied juvenile hormone or anti-JHs, current research shows that ticks cannot synthesize the common insect JHs and have no detectable levels of these hormones in their hemolymph during female reproduction. In ticks, it appears that ecdysteroids, and not JH, regulate expression of the Vg gene and the synthesis and release of Vg protein into the hemolymph. In fact within the Arthropoda, JH has been found only in insects. Methyl farnesoate and not JH regulates Vg synthesis in the Crustacea, the sister group to the insects. Based on this evidence, a new working hypothesis is proposed, i.e., that ecdysteroids and not the JHs regulate vitellogenesis in the Acari including both ticks and mites. To the present, the role of neuropeptides in the regulation of female reproduction in mites is not known.

1. Introduction

The regulation of female reproduction in insects is mainly about the regulation of the synthesis of the yolk protein, vitellogenin (Vg). For most insects that have been studied so far, vitellogenin synthesis is regulated by juvenile hormone (JH) (Gilbert et al. 2000). The JHs are sesquiterpenoids containing a C-1 methyl ester on one end and C10,11 epoxide on the other. Insects have a number of different JHs, i.e., JH0, JH I, JH II, JH III, JH III bisepoxide, and others, depending on the species and developmental stage (Bellés et al. 2005). The presence of high circulating levels of JH in the female adult hemolymph initiates Vg synthesis in the fat body, Vg secretion from the fat body into the hemolymph, and its uptake into eggs in the ovary; once in the eggs, the protein is called vitellin (Vn). Vg is a large molecular weight, multimeric lipoglyco-storage protein synthesized by the fat body and secreted into the hemolymph of females at the time of egg maturation (Sappington and Raikhel, 1998; Hagedorn and Kunkel, 1979). In most insects studied so far, there are multiple Vg genes in each organism (Tufail and Takeda, 2008).

There have been a number of recent advances in our understanding of reproduction in other arthropods, especially in ticks (Acari: Ixodida). These studies include advances in understanding the molecular biology of the tick Vgs, their site of synthesis and role in heme sequestration, and characterization of the Vg-receptor in the ovary (Thompson et al. 2007, Mitchell et al., 2007). Studies have also been conducted to characterize JH in ticks, study the male regulation of female reproduction by peptidic pheromones, and determine the

mechanism for the hormonal regulation of the Vg gene (Neese et al., 2000; Thompson et al., 2005, Donohue et al., 2009b). In mites (Acari, excluding Ixodida), there have been a number of studies on the life history of reproduction but minimal research on the characterization of Vg and the role of hormones in its regulation. However, the general model that has developed is that mites regulate their reproduction as in most insects by the presence and absence of JH. In light of recent new information on the molecular endocrinology of vitellogenesis in ticks and our understanding of the regulation of reproduction in insects and crustaceans, it seems prudent at this juncture to review for the first time our understanding of the same in mites and to re-examine the hypothesis that mites are like insects and regulate female reproduction with JH.

2. Regulation of vitellogenesis in the Mandibulata

Most of our current knowledge on the regulation of vitellogenesis in arthropods is based primarily on studies of insects. In most insects, JH synthesized by the corpora allata is responsible for the initiation of Vg synthesis in the fat body (Fig. 1), secretion of Vg into the hemolymph and uptake by the oocytes through Vg-receptors. Vg deposited in the developing oocyte, called vitellin (Vn), constitutes the major yolk protein. It has been shown in some insects including *Drosophila melanogaster* and *Rhodnius prolixus*, that the follicle cells of the ovaries are also capable of Vg synthesis (Yin et al., 1990, Melo et al., 2000). The general model that JH regulates vitellogenesis does not apply to all insects, in particular in the

Diptera. In mosquitoes, JH seems to condition the oocytes and the fat body to be receptive to ecdysteroids like 20-hydroxyecdysone (20E); 20E consecutively initiates Vg synthesis in the fat body (Fuchs and Kang, 1981) (Fig. 1). Studies with *D. melanogaster* also have shown that JH and ecdysteroids can stimulate the synthesis of Vg in the fat body, but that 20E is required for the uptake of the yolk protein by oocytes (Richard et al., 1998). Studies with the black blowfly *Phormia regina*, suggested that ecdysteroids regulate Vg synthesis, and the uptake of Vg by the oocytes occurs in response to JH (Yin *et al*., 1990). Exceptions to the general JH model also occur in other groups including Lepidoptera and Hymenoptera. For instance, in the fall army worm, *Spodoptera frugiperda*, JH promotes the production of 20E that in turn initiates Vg synthesis in the fat body, while JH is required for Vg uptake into oocytes (Sorge et al., 2000). However, in most insects, a high level of circulating JH is the principal trigger that initiates vitellogenesis. In honey bees, it appears that JH does not have a clear function during vitellogenesis, as the JH titers in queens are low; in fact, 20E titers are high in queens, thus suggesting a role for ecdysteroids during honey bee vitellogenesis (reviewed by Robinson and Vargo, 1997).

In the Crustacea, the immediate precursor of JH III, methyl farnesoate (MF), appears to regulate vitellogenesis (Laufer et al., 1987b). The mandibular organ (MO) of crustaceans has been identified as the major site of synthesis of MF (Laufer et al., 1987a). A strong, positive correlation has been observed between MF and vitellogenesis in the spider crab, *Libinia emarginata*, and in other crustaceans (Laufer et al. 1987a, b). In turn, the role of ecdysteroids during vitellogenesis in crustaceans is not yet clear. Ecdysteroids, which are

synthesized in the Y-organ, remain low throughout vitellogenesis in the hemolymph of *L. emarginata* (Laufer *et al*. 1988). In some species, ecdysteroids seem to have a major role in Vg synthesis but in others may have only a minor role or have no defined function at all (Subamoniam, 2000; Charniaux-Cotton, 1985). In some instances, ecdysteroids may be necessary for Vg absorption into oocytes (Charniux-Cotton, 1985). The presence of ecdysteroids in adults may be unrelated to egg development since some crustaceans periodically molt during the adult stage (Subramoniam, 2000).

3. Regulation of vitellogenesis in ticks

Within the Acari, the Ixodida (ticks) has been so far the best studied group relative to the hormonal regulation of vitellogenesis. Mating and blood feeding to repletion are necessary for yolk protein synthesis in the American dog tick, *Dermacentor variabilis* (Thompson et al. 2005) and others. Vg is synthesized primarily in the fat body and midgut, and to a lesser extent in the ovary (Rosell and Coons, 1992; Thompson et al. 2007). Indeed, Thompson et al. (2007) clearly stated that they could not discard contamination by fat body as the reason they observed Vg message in the ovary of *D. variabilis*. Pound and Oliver (1979) showed that precocene 2, an anti-JH, interfered with the oviposition of the soft tick, *Ornithodoros parkeri*, and that exposure of exogenous JH III could in part restore egg production. This and other pharmacological studies (reviewed by Roe et al. 2008) suggested that the regulation of vitellogenesis in ticks might be in response to JH, in the same manner

as it occurs in insects. Other research, however, contradicted this view point. For example, Taylor *et al*. (1991) found that topically applied JH I, II, III and the JH-analog methoprene did not initiate Vg synthesis in *O. parkeri* and concluded that JH does not regulate vitellogenesis in this species. JH radiosynthesis assays, electron ionization gas chromatography-mass spectrometry (EI GC-MS) and the *Galleria* JH bioassay, demonstrated that the hard tick, *D. variabilis*, and the soft tick, *O. parkeri*, were not able to synthesize JH I, II, III, III bisepoxide or MF and did not contain any compounds with insect JH activity (Neese et al. 2000). Sankhon et al. (1999) showed that 20E but not JH would increase the concentration of Vg in organ culture of *D. variabilis* fat body. Thompson et al. (2005) found that 20E injected into part-fed, virgin females of *D. variabilis* attached to the host initiated vitellogenesis, increased the levels of the protein in the hemolymph and caused Vg uptake into the oocytes, while JH had no effect. The ovaries of these 20E-injected, part-fed (virgin) females reach weights similar to that of mated, replete females. These studies suggest that ticks are not like most insects and regulate egg development with ecdysteroids and not JH. Seixas et al. (2008) and Friesen and Kaufman (2004) showed that although 20E alone can increase Vg titers in the hemolymph in the hard tick, *Amblyomma hebraeum*, Vg uptake by the oocytes requires a yet unknown vitellogenin uptake factor (VUF) found in the hemolymph of replete females (Seixas *et al*. 2008). Although additional work is still needed, it is clear in the few ticks that have been studied that 20E is responsible for the initiation of Vg synthesis. Ecdysteroids in ticks are secreted by the epidermis and converted into 20E by the fat body (Zhu et al., 1991).

4. Life history of reproduction in mites (excluding ticks)

The great diversity in habitats and habits of mites (excluding ticks) is reflected in their range of different reproductive strategies, which include both sexual and asexual reproduction, with arrhenotoky, pseudoarrhenotoky and thelytoky (Norton et al. 1993). These reproductive strategies occur throughout the different groups or suborders of the Acari (Fig. 1), and in some instances, strains of the same species have different modes of reproduction (Hughes, 1969).

Sexual reproduction, also known as diplodiploidy, is known to occur in the Mesostigmata (Norton et al., 1993), Prostigmata (Shirai et al., 1984), Oribatida (Norton et al. 1993) and Astigmata (Heinemann and Hughes, 1970). In this form of reproduction, male and female offspring are biparental and diploid. Diplodiplidy provides the advantage of a higher genetic variation. However, arrhenotoky is the most widely distributed reproductive strategy in the Acari (Norton et al. 1993; Hughes, 1969). In this type of asexual reproduction, unfertilized eggs give rise to haploid-male offspring while fertilized eggs become diploid-females. Although first thought to be arrhenotokous because males are haploid and females are diploid, it was observed that mites from the family Phytoseiidae require mating in order to lay eggs (Hoy, 1979). Further studies showed that male phytoseiid mites start embryogenesis as diploids but lose the paternal set of chromosomes by heterochromatization or chromosome elimination (Nelson-Bees et al. 1980; Hoy, 1979). This type of reproduction is called pseudoarrhenotoky and is currently known to be particular to the families

Phytoseiidae and Otopheidomenidae (Mesostigmata; Norton et al., 1993). Thelytoky is less common in the Acari. This kind of reproduction has been observed in some species of the Mesostigmata (Uropodidae; Norton et al., 1993), Prostigmata (Tenuipalpidae; Pijnacker et al., 1980), Oribatida and Astigmata (Norton et al., 1993). In thelytoky, female haploid mites arise from unfertilized eggs while males are not known or the few that develop do not mate or produce non-viable sperm (Norton et al., 1993).

With diverse life histories and reproductive strategies, it should be expected that multiple intrinsic factors must play a role in the regulation of vitellogenesis in mites. For example, nothing is known about male factors transferred during copulation, which in other groups including insects and ticks can stimulate ovarian development, increase oviposition and reduce female longevity (Sirot et al., 2008; Kaufman, 2004; Wolfner, 1997). For instance, ticks require copulation and feeding to repletion to complete vitellogenesis (Thompson et al. 2007), and it has been suggested that peptide pheromones (i.e. engorgement factors, EF) transferred from the male to the female during copulation stimulate the female to feed to repletion, a process necessary to carry out vitellogenesis. Weiss and Kaufman (2004) found that the action of two combined peptides, EFα and EFβ (called voraxin), transferred from male *Amblyomma hebraeum* to part-fed females, were necessary for females to feed to repletion and for ovarian development. Donohue et al. (2009b) found a homologous sequence of EFα, but not of EFβ, in a male *Dermacentor variabilis* reproductive system high-throughput sequencing analysis. In fact, only EFα homologous sequences have been identified for other tick species including *Ixodes scapularis* and *Rhipicephalus microplus*, in

protein and nucleotide sequences available in the NCBI database (Donohue et al., 2009b). However, while Donohue et al. (2009b) were able to knock down the expression of EFα in male *D. variabilis* through RNA interference (RNAi), part-fed females allowed to mate with such males were still able to fully engorge, thus showing no direct stimulatory effect of EFα in this species. The RNAi approach to further evaluate the function of voraxin (EFα and EFβ) on *A. hebraeum* yielded unexpected results, showing no reduction in gene expression, and female ticks that mated with voraxin-dsRNA treated males fed to repletion (Smith et al., 2009). Although it is not completely understood in ticks how male pheromones may exactly affect physiological changes in the female, these changes occur. Thus, it is reasonable to expect that in mites with diplodiploidy and pseudoarrhenotoky reproduction, where copulation is necessary for reproduction, males may transfer peptides and/or other factors to the female that may be critical to female reproduction. This has not yet been studied in mites.

5. Mite female reproductive system and yolk proteins

5.1 Histological studies of the female reproductive system and oogenesis

The diversity of the Acari is apparent in an examination of the female reproductive system. The mesostigmatid, prostigmatid and oribatid mites have an unpaired ovary (Alberti and Zeck-Kapp, 1986; Mothes-Wagner and Seitz, 1984; Witaliński, 1986) while astigmatid mites possess paired ovaries (Lekimme et al., 2005). Mesostigmatid and prostigmatid mites have a single oviduct that connects the ovary to the vagina (Mothes-Wagner and Seitz, 1984;

Matsubara et al., 1992). In contrast, although oribatid mites have an unpaired ovary as well, they have paired oviducts that converge to the vagina (Heethoff et al., 2007). A spermatheca or receptaculum seminis is present in prostigmatid and astigmatid mites (Mothes-Wagner and Seitz, 1984; Feiertag-Koppen and Pijnacker, 1982; Lekimme et al. 2005), but is not present in mesostigmatid nor oribatid mites (Toyoshima et al., 2000; Alberti and Zeck-Kapp, 1986; Heethoff et al. 2007). Female mites seem to lack apparent accessory glands (Alberti and Zeck-Kapp, 1986; Pijnacker et al., 1981), but in some prostigmatid mites secretions in the oviduct have been observed (Matsubara et al., 1992), and in mesostigmatid mites a glandular wall is present in the oviduct (Toyoshima et al., 2000). Furthermore, besides having oviducts, astigmatid mites have a glandular uterus that connects to the oviparous (oviposition opening) (Lekimme et al. 2005). It remains to be determined whether the oviduct has accessory gland-like functions. Mesostigmatid mites have associated with the ovary, the lyrate organ, which provides support and nutrition to developing oocytes (Alberti and Zeck-Kapp 1986). It is located ventral to the ovary and has two lateral lobes; the ovary and lyrate organ are connected through a series of nutritive cords (De Ruijter and Kaas, 1983; Alberti and Zeck-Kaap, 1986).

Mite oocytes, as is the case for other arachnids, develop enclosed by a thin membrane (Mothes-Wagner and Seitz, 1984; Steiner et al., 1995), which during late stages of vitellogenesis is displaced by microvilli (Witaliñski, 1986). Prostigmatid mites have meroistic ovaries, where the oocytes are attached to multinucleated nurse cells thought

cytoplasmic bridges (Kawakami et al. 2009; Feiertag-Koppen and Pijnacker 1982; Pijnacker et al., 1981).

Some studies suggest that during early stages of vitellogenesis yolk proteins are synthesize by the oocytes (endogenous) and as vitellogenesis progresses, the majority of the yolk (Vg) is absorbed from the hemolymph (Shatrov, 1997). In the twospotted spider mite *Tetranychus urticae*, yolk protein synthesis by the oocyte seems to occur throughout vitellogenesis but remaining at low levels (Mothes-Wagner and Seitz, 1984). In the microtrombidiid mite, *Platytrombidium fasciatum*, protein synthesis within the oocyte appear to be the primary source of yolk protein in this species (Shatrov, 2002). In mites with meroistic ovaries, as is the case of the prostigmatid mites, the oocytes are connected to the nurse cells that provide cytoplasm and other organelles (Mothes-Wagner and Seitz, 1984; Matsubara et al., 1992). The nurse cells have a higher content of proteins, and thus some authors suggest they could also provide proteins to developing oocytes (Feiertag-Koppen and Pijnacker 1982); however, is not clear if these proteins are yolk proteins.

The source of extraovarian Vg has not been clearly determined for any mite species and it remains to be addressed. Although in ticks the fat body is one of the major tissue sources of Vg (Thompson et al., 2007), most mites lack this tissue (Filimonova 2001, Shatrov 1998, Mothes-Wagner and Seitz 1984). Gamasid mites may have specialized cells that could have some of the same function as the fat body; this is the case of the phytoseiid mites, *Phytoseiulus persimilis* and *Typhlodromus rhenanoides*, that have enlarged cells beneath the

epidermis and along the gut caecae. Di Palma and Alberti (2001) suggested that these cells may substitute for fat body function and may be implicated in providing yolk to developing oocytes. Steiner et al. (1995) observed that *Varroa jacobsoni* oocytes absorbed exogenous vitellogenin from the hemolymph, but the exact site of synthesis could not be determined. Besides ticks and some mesostigmatid mites, the other groups in the Acari seem to lack fat body. In the hemolymph of the trombiculid mite *Hirsutiella zachvatkini*, specialized cells called nephrocytes are present that are thought to be the remnants of fat body (Shatrov, 1997). However, these cells seem not to be involved in Vg synthesis due to their lack of rough endoplasmic reticulum necessary for protein synthesis (Shatrov 1997). With the absence of the fat body, other tissues have been suggested as possible sites of Vg synthesis. The oocytes of *T. urticae*, *T. harti*, *H. zachvatkini,* and *P. fasciatum* are in close association with midgut cells, and this tissue has been suggested as a source for Vg (Mothes-Wagner and Seitz 1984; Shatrov, 1997; Shatrov 2002). Indeed, in *T. harti*, bridges have been observed between the oocytes and the midgut (Matsubara et al. 1992). Thus, the role of the midgut during vitellogenesis should be further investigated. Interestingly, the midgut was shown to produce the Vg message in the American dog tick (Thompson et al., 2007). Uptake of extraovarian Vg from the hemolymph in mites appears to take place through pynocytosis, aided by the formation of microvilli around the oocyte (Shatrov, 1997; Shatrov, 2002). Vg-receptor mediated pynocytosis occurs in ticks, but there is no information yet on Vg-receptors (VgRs) in mites. Mitchell et al. (2007) found in *D. variabilis* that suppression of the VgR message in replete, mated females from injections of $dsRNA_{VgR}$ repressed the

development of mature eggs, highlighting the critical function of the receptor in tick reproduction. Similar studies are needed in mites to better understand both the sites of Vg synthesis and their mechanism of uptake.

During vitellogenesis, the oocytes in mites increase in size due to the absorption of cytoplasm, organelles and nutrients including Vg. *Varroa jacobsoni* oocytes enlarge 25 times their volume (Steiner *et al.* 1995) while oocytes of the spider mite, *T. urticae*, increase 10-fold during the process of vitellogenesis (Mothes-Wagner and Seitz, 1984). In *Psoroptes* spp., the oocytes can reach 90 – 150 μm diameter, and electron microscope observations estimated that mature yolk granules are 0.3-0.9 μm in diameter (Lekimme et al. 2005). Bigger yolk granules are observed in *V. jacobsoni* reaching 9 μm in diameter (Steiner et al. 1995), and in the case of *P. fasciatum*, mature yolk vesicles can be 10-20 μm in diameter (Shatrov, 2002). The process of yolk accumulation can be fairly rapid, as in the case of *T. urticae* where the expression of Vg starts at day 1 after female adult eclosion (Kawakami et al. 2009). In the case of *P. persimilis*, the accumulation of yolk granules starts around 12 h after copulation (Toyoshima et al., 2000). Other nutrients are also deposited into the oocyte during vitellogenesis including lipids and carbohydrates (Mothes-Wagner and Seitz, 1984; Shatrov 1997). In the case of *P. fasciatum*, it was observed that the amount of lipid is about the same as yolk protein (Shatrov, 2002). However, the exact composition of lipids and carbohydrates has not been determined yet for any mite species. After vitellogenesis has taken place, the oocytes form the vitellogenic envelop or chorion, and enter the oviduct lumen for oviposition (Witaliñski, 1993).

5.2 Yolk Protein

Currently, our understanding of the Vn in mites at the protein level is limited to a single species. Cabrera et al. (2009) characterized Vn from the twospotted spider mite, *T. urticae*, in comparison to that from the American dog tick. The mite Vn on 4-12% tris-glycine, native PAGs was 476 kDa which was smaller than that of 590 kDa for *D. variabilis* (Fig. 2). Our best understanding of Vg within the Arthropoda is with insects, where Vg is a phosphoglycolipo-protein ranging in size from 200 to 700 kDa (Sappington and Raikhel, 1998). In the Crustacea, Vg and Vn are lipoglyco-carotenoproteins from 300 to 500 kDa (Lee et al., 1997). Less is known about these proteins in the Acari, and the vast majority of research is with ticks (reviewed by Roe et al., 2008; Gudderra et al., 2002b). In *O. moubata* and *O. pakeri*, for example, Vn was reported with a molecular weight of 600 kDa (Chinzei et al., 1983; Taylor and Chinzei, 2001). In *D. variabilis*, the size range for Vg was 320-486 kDa (Sullivan et al., 1999; Gudderra et al., 2001, 2002a,b; Thompson et al., 2005, 2007) and for Vn, 370 to 480 kDa (Rossell and Coons, 1991). The reason for these reported differences in size is not known but Cabrera, Khalil and Roe (unpublished) and as discussed in Cabrera et al. (2009) found that estimates in the size of Vn from *D. variabilis* was more associated with the commercial source of the protein standards used to determine the molecular weight than the method of native PAGE. The size of the twospotted spider mite reported by Cabrera et al. (2009) was within the range of reported sizes for tick yolk proteins but smaller than that from the American dog tick (Fig. 2). Thompson et al. (2007) found that the conceptual sequence derived from the Vg1 message of *D. variabilis* was smaller by about one-half from

that resolved by electrophoresis, suggesting that the protein in hemolymph and in the egg may exist as a dimer. Considering the size of Vn from *T. urticae* was also greater than the molecular weight of the conceptual *D. variabilis* Vg1 protein by a factor of more than two fold and what we know also about insect Vgs, it appears likely that the mite yolk protein is also oligomeric. More work will be needed in the future to confirm this hypothesis.

Although native spider mite Vn migrated as a single band on native PAGE, isoelectric focusing resolved five proteins with pI values slightly acidic to neutral (pH 5.8, 6.2, 6.7, 7.0 and 7.2) (Cabrera et al., 2009), similar to that reported before for insects (Raikhel and Dhadialla, 1992). Ticks have multiple yolk protein messages (Roe et al., 2008; Donohue et al., 2009a), and Kawakami et al. (2009) found four different partial Vg messages from *T. urticae* (discussed in more detail later). Multiple Vgs have also been found in insects and other arthropods (Tsang et al., 2003; Roe et al., 2008; Donohue et al., 2009a), and these proteins appear to share a common ancestry with the acarines (Roe et al., 2008; Donohue et al., 2009a). The isoelectric focusing results suggest that the multiple Vg messages found in *T. urticae* by Kawakami et al. (2009) are being translated into protein found in the mite egg.

Boctor and Kamel (1976) estimated the lipid and carbohydrate content in tick Vg was 5.5-8.5% and 4.5%, respectively. Recently, Cabrera et al. (2009) also found that *T. urticae* Vn bound lipid and carbohydrate but proportionally less relative to BioRad and 280/260 protein estimations as compared to the same for *D. variabilis* Vn. It is also interesting that ticks which are obligate blood feeders apparently have lost the ability to synthesize heme

(Braz et al. 1999) and have developed special storage proteins including Vg that bind heme (Donohue et al., 2008, 2009a; Roe et al., 2008). Some of these heme binding storage proteins may also be found in the black widow spider (reviewed by Donohue et al., 2009a), but it appears that Vn in the twospotted spider mite does not bind heme (Fig. 3)**.** This suggests a fundamental difference exists within the Acari between blood feeding and non-blood feeding mites relative to heme synthesis and processing. Comparative studies between these two groups in respect to the structure of the Vgs could be useful in identifying the motif(s) in the tick Vg that are critical for heme binding. Currently there is no information on how Vgs bind heme. Roe et al. (2008) and Donohue et al. (2009a) hypothesized that heme sequestration by proteins like Vg in ticks was critical to the evolution of hematophagy in this group. Studies of heme processing in mites versus ticks could thus be fundamental to the basic understanding of the obligatory move to blood feeding.

5.3 Molecular biology of mite Vg

At present, no complete Vg gene of any mite species has been sequenced or is available in the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/; April 2009). However, partial sequences of four different Vg genes have been confirmed in the spider mite, *T. urticae* (Acari: Prostigmata) (Kawakami et al. 2009). These sequences range from 1072 to 1155 bp long (Accession numbers AB455063, AB455064, AB455065, AB455066). The work of Kawakami et al. (2009) is the first to show

the presence of multiple Vg messages in the same mite species; the presence of multiple tick Vg messages was recently reviewed (Roe et al., 2008). It is not confirmed yet whether the multiple Vg messages in *T. urticae* are all translated into protein but this would be an obvious expectation (discussed in more detail later). Twenty eight additional mite ESTs (incomplete full messages) were identified in the NCBI for the first time as part of this review which are similar to known Vg sequences of other arthropods (Table 1). These ESTs are from six different mite species, i.e., *Blomia tropicalis* (10 ESTs), *Dermatophagoides farinae* (13), G*lycyphagus domesticus* (3), *Sarcoptes scabiei* (2), *D. pteronyssinus* (1), and *Suidasia medanensis* (1), within the Astigmata and in some cases might represent additional evidence of multiple Vg genes in the same mite.

Since there are no current full-length nt or protein sequences for Vg from mites, it is informative to discuss Vgs from ticks (Acari: Ixodida) relative to our limited understanding of the same in mites. The first full length Vgs were obtained from the American dog tick (Fig. 4). The tick Vgs have three functional domains as described before by Donohue et al. (2009a), i.e., the lipoprotein N-terminal domain, a domain of unknown function (DUF1943) and the von Willebrand type D domain as shown in Fig. 4. One of the challenges in the assignment of function to these proteins in ticks is the presence of other non-Vg storage proteins that contain similar domains in approximately the same locations, e.g. the tick carrier protein, CP (Donohue et al., 2009a). The identification of proteins analogous to CP in ticks has not yet been identified in mites, but it is likely that constitutively expressed storage proteins common in the Arthropoda in general will also be found in mites. As part of this

review, alignments were conducted between Vg1 and Vg2 from *D. variabilis* and the *T. urticae* Vgs and between Vg1 and Vg2 from *D. variabilis* and all of the putative Vg ESTs using the program Vector NTI (Invitrogen) (in Table 1). All of the alignments occur toward the C-terminus of the tick Vg in the von Willebrand type D domain**.** Some but not all of the ESTs in Table 1 were also identified as being part of this domain using tools in the NCBI website. Each EST sequence was submitted to the open reading frame (ORF) finder and using the translation for each of the possible six ORF, each putative peptide was compared to the available sequences in the protein data base (blastp)**.** We chose the translated peptide with the corresponding ORF with similarities to arthropod and non-arthropod vitellogenin to conduct alignments. We considered ESTs with an e-value of $1e^{-5}$ or lower (probability of the EST being more similar to other sequences in the NCBI database), because of the short length of the sequences. Alignments between *D. variabilis* Vg1 and selected mite sequences and between *D. variabilis* Vg2 and the same selected mite sequences are shown in Fig. 4. With an incomplete understanding of the sequence of other mite storage proteins and/or other mite proteins that may also contain the von Willebrand type D domain, the challenge now will be the correct assignment of function to these putative mite Vgs. Probably the most straight-forward approach is to simply look for female-specific expression.

Most insect vitellogenin proteins have a cleavage site which generates two or more protein subunits (Tufail and Takeda, 2008; Sappington and Raikhel, 1998). This cleavage signal, RXXR, was found once in Vg1 and five times in Vg2 in the American dog tick (Thompson et al., 2007; illustrated also in Fig. 4). This cleavage site (RTVR for Vg1-Vg3

and RSIR for Vg4) was also found once in the conceptual sequence of the *T. urticae* partial Vgs identified by Kawakami et al. (2009). In *D. variabilis* Vg1, the predicted molecular weights after cleavage were 49.5 and 157 kDa, and Cabrera et al. (2009) found corresponding bands at 35.0-56.6 and 168.4 kDa, respectively, for Vn from the same tick species resolved by SDS-PAGE. There were also corresponding bands from newly oviposited eggs from *T. urticae* at 176.5 and 43.4 kDa, suggesting the cleavage sites identified by Kawakami et al. (2009) produce the expected post-translational products (Cabrera et al., 2009). In *D. variabilis* and other ticks, Vg and Vn resolved by SDS-PAGE (reviewed by Thompson et al., 2007 and Roe et al., 2008) show additional post-translational processing. Vn from newly oviposited eggs of the twospotted spider mite resolved by the same method was even more complex than that of the American dog tick (Cabrera et al., 2009). This increased complexity may be the result of additional unique protein messages in the mite as compared to ticks, the presence of other cleavage sites and/or artifacts of sample preparation. Thompson et al. (2007) found on SDS-PAGE that Vn from *D. variabilis* appeared as doublets suggesting multiple Vg messages. Cabrera et al. (2009) also found evidence of even a greater number of repeats in *T. urticae*, which supports the findings of Kawakami et al. (2009) of at least four different Vg messages in the same mite species.

6 Endocrine regulation of mite female reproduction

6.1 Direct detection of ecdysteroids and JH in mites

Studies on the identification and physiological role of ecdysteroids and juvenile hormone in mites are minimal. The ecdysteroids so far identified are ecdysone, 20E, 2-deoxyecdysone, and Makisterone A. In the acarid mite, *Tyrophagus putrescentiae*, 2-deoxyecdysone appears to be the most abundant ecdysteroid (Sakagami *et al*. 1992). Because ecdysone and 20E are derived from 2-deoxyecdysone, it was suggested that these hormones may also be present in *T. putrescentiae* but at low levels (Sakagami *et al*. 1992). In other mites like the mesostigmatid *Dermanyssus gallinae*, the protonymphs and deutonymps demonstrated levels of 20E almost 5 times higher than ecdysone (Chambers *et al*. 1996). Feldlaufer and Harfelder (1997) found in the Varroa mite, *V. jaconsoni*, 20E was the most abundant ecdysteroid, followed by ecdysone and Makisterone A. The site of synthesis of the ecdysteroids, developmental regulation of ecdysteroid titer, including changes during female reproduction, and the relative biological activity of the different ecdysteroids so far identified has not been determined.

Although the working hypothesis has been that JH is important in the regulation of mite vitellogenesis, no insect JHs have been identified in mites. There also has been minimal research to examine whether mites can synthesize the insect JHs, to determine by direct detection using high resolution approaches like electron impact-gas chromatography mass spectroscopy whether they have JH, or the use of *Galleria* bioassays to detect JH activity in

crude tissue homogenates from mites. The only evidence of a JH precursor in mites was from the study of Regev and Cone (1975). In this work, farnesol, a precursor of JH, was found by thin layer chromatography, ultraviolet light analysis, and gas liquid chromatography mass spectrometry in whole body homogenates of the deutonymph of the twospotted spider mite (Regev and Cone, 1975). It was observed that farnesol acted as a sex attractant, released by the female spider mite to attract males; this work did not consider the possible role of farnesol in female reproduction. The report is interesting, however, in that it suggests the presence of at least part of the pathway for JH biosynthesis in mites. In further studies, Regev and Cone (1976) showed that topically applied farnesol increased oviposition in the twospotted spider mite. However, it is also worth noting that farnesol is present in plants as well, and Regev (1978) observed that strawberry plants with higher levels of farnesol were more susceptible to damage by the carmine spider mite, *T. cinnabarinus*, compared with varieties with lower levels of the compound. It is possible that plants could be the source of farnesol in spider mites, and plant farnesol is released by the female to attract males.

6.2 Effect of JH, anti-JHs and JH analogs on mite reproduction

Probably the most convincing evidence that mites might have JH and use JH for the regulation of female reproduction was the study of Oliver et al. (1985). In this study, female mesostigmatid mites, *D. gallinae*, were treated with the anti-JH compound, precocene 2 (P2);

female mites were placed in glass vials, and P2 was applied to either the lid or a filter paper circle glued to the lid. Results showed that mites exposed to P2 had a reduced number of progeny at the seventh day after treatment, compared to the controls, and that there was an inverse correlation between the time of exposure and P2 dosage with the number of offspring (Oliver et al., 1985). Interestingly, treatments with JH III could partially rescue the P2 effect on reproduction, thus suggesting that JH was the mite gonadotrophic hormone. While these results are not direct evidence of the presence of JH in mites, they are intriguing. Unfortunately, there have not been other complementary studies that could offer a better understanding of the precise effect of P2 and JH on mite physiology. It should be pointed out that similar results were observed previously in a comparable study with ticks (Pound and Oliver, 1979). In soft ticks, *Ornithodoros parkeri*, topically applied P2 reduced the number of eggs laid per female, while in rescue experiments, ticks that were exposed to P2 and then JH III were able to produce eggs (Pound and Oliver, 1979). However, the restoration of female reproduction occurred at the low JH dosage (1 μg) and not at the high JH dosages (10 and 100 μg) tested. Even though these results are difficult to explain, the effects of P2 and JH III on tick and mite reproduction could have other explanations beside hormonal regulation, such as an effect of toxicity or hormoligosis (the stimulation of female reproduction by sub-lethal doses of toxic compounds). In fact, hormoligosis has been observed before in ticks when treatment with the pyrethroid insecticide, cypermethrin, resulted in increased Vg synthesis in *O. parkeri* (Taylor et al., 1991).

There have been a number of studies on the treatment of mites with JH, JH precursors and JH mimics and examining the effect of these treatments on development, egg production, population growth and mortality. For the most part the studies are contradictory or not informative in determining whether mites have JH or use JH to regulate reproduction. Two studies were conducted on the effect of farnesol on mite reproduction (Table 2). Regev and Cone (1976) topically applied farnesol to females of *T. urticae* and found the number of eggs oviposited per female increased over that of the control by 5 to 46%. In contrast, female *Tyrophagus putrescentiae* treated with farnesol oviposited a similar number of eggs as compared to the control (Czaja-Topinska et al. 1979). These results were equivocal in determining the role of farnesol in mite reproduction.

The role of JH III in mite reproduction was also examined in two mite species. Hänel (1983) found that JH III applied to bee larvae increased the number of offspring per female of the varroa mite, *V. jacobsoni*, parasitizing the larvae. This study found a positive correlation between JH III levels in the bee larva and the number of varroa mite offspring. Furthermore, it was shown that treatment of winter bees, which typically have low levels of JH III, with exogenous JH III increased the percentage of varroa mites that produced offspring (39% of mite produced offspring) compared to mites parasitizing untreated winter bees (8% of mites produced offspring) (Hänel and Koeniger, 1986). Several explanations might explain these results which include (a) an exclusive effect of JH III on bee physiology which enhances mite development and reproduction indirectly, (b) a direct effect of JH III on

mite development and reproduction, or (c) a combination of both. Further studies will be needed to clarify the role of host JH III levels on *V. jacobsoni* reproduction.

There have been a number of studies on the effect of JH agonists on a diverse group of mites. In general these studies show that JH analogs have a detrimental or no effect on mite fecundity (Table 2). For instance, Czaja-Topinska et al. (1979) evaluated 56 JH analogs and found that most of them, including methoprene, were not lethal and did not interfere with the fecundity of the acarid mite, *T. putrescentiae*. In contrast, methoprene showed detrimental effects on the reproduction of the mesostigmatid, predatory mite, *Amblyseius brazilli*, by reducing the number of eggs oviposited per female and increasing the preoviposition period (El-Banhawy, 1977). In further studies, El-Banhawy (1980) not only confirmed previous observations with *A. brazilli*, but also showed methoprene reduced the number of eggs per female per day of the spider mite, *Tetranychus desertorum*. Methoprene and hydroprene, also a JH analog, reduced the population growth of the house dust mite, *D. farinae*, although it was not clear if this was due to lethal effects or to interfering directly with reproduction (Downing et al. 1993). In a similar study, Downing et al. (1990) also found that the number of *D. farinae* mites was lower on arenas treated with methoprene, hydroprene and fenoxycarb (a carbamate with JH-like effects). Downing et al. (1990, 1993) noted in these studies that the dose needed to produce lethal effects in mites was 25- to 67-times higher than the recommended rates for flea control for these compounds. Likewise, Mazyad and Mohammad (2006) also found methoprene and hydroprene suppressed population growth of *D. farinae*, applied topically and through the diet, noting that the

dosages used were also high. Although no information on reproduction was obtained, methoprene applied at recommended rates for the control of the sciarid fly, *Lycoriella solani*, did not harm populations of the predatory mite, *Hypoaspis miles* (Ali et al. 1999). Another JH analog, S-kinoprene, showed no significant lethal effect on the predatory mites, *P. persimilis* and *A. fallacies.* The treatment slightly reduced the mean percentage of female *P. persimilis* mites laying eggs and the number of eggs laid per female, but did not affect *A. fallacies* reproduction (Bostanian and Akalach, 2006).

Contrasting results have been obtained from the evaluation of fenoxycarb on mite reproduction (Table 2). A stimulatory effect on the population growth of the grain mite, *Acarus siro*, was observed when fenoxycarb was incorporated into the food source, increasing egg production by 43 to 99% compared to the control (Thind and Edwards 1990). Although not tested for its effect on the reproduction of the predatory mite, *P. persimilis*, fenoxycarb was harmless to this commonly used biological control agent (Oomen et al., 1991). Moreover, fenoxycarb and another JH analog, epofenonane, were relatively safe and posed a low risk to predatory mites in apple orchards (de Reede et al. 1984). Thus, is not clear if the increase in population growth observed in *A. siro* by Thind and Edwards (1990) was due to a potential JH mimic induction of mite reproduction or if it was a result of sublethal effects causing hormoligosis.

Other JH analogs have been examined for their effects on mite reproduction (Table 2). For example, CGA 29'170 and ZR-777 decreased the number of eggs oviposited per

female of the spider mite, *T. urticae*, and the predatory mite, *P. persimilis* (Klein-Koch, 1976). Pyriproxyfen, another highly potent JH mimic, was only lethal to nymphs of the storage product mite *T. putrescentiae* at high dosages (≥10,000ppm); adult mortality and number of eggs laid per female was not significantly reduced by this JH analog, compared to the control treatment (Sánchez-Ramos and Catañera, 2003). Moreover, when the predatory mites, *Neoseiulus fallacies*, were exposed to pyriproxyfen, there was no reduction in the number of eggs laid per female (Villanueva and Waldenbach, 2005). Cabrera et al. (2004) found that pyriproxyfen did not change the duration of the oviposition period, change the number of eggs oviposited per female or affect the viability of oviposited eggs of the predatory mite, *Stratiolaelaps scimitus*.

In toto, studies with anti-JHs, precursors to JH, JH III and JH mimics were equivocal in establishing that mites have JH or use the insect JHs to regulate reproduction, metamorphosis or development in general. These compounds were not exceptional miticides at low doses, and the effects on reproduction ranged from no effect to reduced or enhanced egg production to varying degrees associated with different levels of mortality. There were no reports of typical JH mimic effects such as super-numery molting, enhanced feeding and increases in the size of the mite above normal, molting to intermediate stages or intermediate stage mortality as would be expected for the treatment of insects with JH or JH mimics. In conclusion, there is no evidence that supports the presence of JH or JH-like compounds in mites and ticks. However, more studies are needed to examine this question. Furthermore, research is needed to examine if earlier steps in the JH pathway are present in the Acari.

6.3 Ecdysone analogs and antagonist

A few studies have been conducted to examine the potential effect of ecdysone mimics or antagonists on mite development and reproduction. Sánchez-Ramos and Castañera (2003) showed that the ecdysone analog, halofenozide, produced lethal effects on both the immature and adult stages of *T. putrescentiae*, and it reduced significantly the number of eggs ovipoisted per female (Table 2). Because the treatments were producing mortality in adults, it is not clear whether the reduction in the number of eggs oviposited is a result of direct interference with female vitellogenesis, egg maturation and/or oviposition or alternatively indirectly associated with the mechanism of toxicity. Villanueva and Walgenbach (2005) showed that the ecdysteroid agonist, methoxyfenozide, was not toxic and did not affect the oviposition rate of the predatory mite, *Neoseiulus fallacies*, at field recommended rates in apple orchards. Although methoxyfenozide was not evaluated for its effect on reproduction, Laurin and Bostanian (2007) found that the insecticide was not toxic to the predatory mite, *Anystis baccarum*, under laboratory conditions, even at 32 times the recommended rate for apple orchards. The ecdysone agonist tebufenozide is considered to be compatible with biological control agents due its low toxic effect on predatory mites, i.e. *Amblyseius womersley* (Mochizoki, 2003) and *Typhlodromus pyri (*Hardman et al. 2000). Finally, treatment with the ecdysone antagonist, diofenolan, increased the number of eggs oviposited per female in the mesostigmatid mites, *Euseius addoensis and E. citri*, and produced moderate levels of mortality (Grout et al., 1997). No other reports on the effects of ecdysone analogs and antagonists on mite reproduction were found in the literature.

7. New hypothesis for the regulation of female reproduction in mites: A unifying model for the Acari.

The general view has been that mites are like insects and regulate vitellogenesis with the JHs. However, there is no direct evidence that mites can synthesize the insect JHs, have JH in their tissues or even produce any compound that would have JH activity in an insect *Galleria* bioassay. For the most part, there has been essentially no effort to obtain direct evidence of JH in mites. A number of studies have examined the pharmacological effects of JH precursors, JH, anti-JHs and JH mimics on mite reproduction and mortality; these data could be fairly summarized as not supporting a role for JH in the regulation of mite female reproduction. The idea that mites may not have JH should not be too surprising because direct detection methods for the identification of JH in ticks as well as analogous pharmacological studies in ticks with JH, anti-JHs and JH mimics were unsuccessful in demonstrating a role for JH in the Ixodida. The view that JH is not in mites or ticks also should not be surprising since even the sister group to the Insecta, the Crustacea do not have JH. They use the precursor to JH, i.e., methyl farnesoate, to regulate vitellogenesis, but apparently are unable to add the C10,11 epoxide to produce JH. Based on simply an evolutionary argument for the Arthropoda, it would be assumed that the Acari do not have JH or use JH to regulate female reproduction.

A unifying model to describe the regulation of vitellogenesis and female reproduction in the Acari is shown in Fig. 5. The results from this review suggest that the common insect

JHs are not involved in the initiation of yolk synthesis in both mites and ticks (Fig. 5). However, this is not to say that earlier parts of the JH synthesis pathway are not found in the Acari or that the precursors of JH have no role in Acari development. To the contrary, our view is that the pathway is likely present up to a point and has some function in acarine physiology. Research is needed to validate this hypothesis. Unpublished data by Roe, Donohue, Khalil and Sonenshine in the analysis of a synganglion 454 transcriptome from the American dog tick female adult discovered most of the putative enzymes leading to the synthesis of farnesyl-PP. The JH and sterol branches were absent.

Our current working hypothesis is that ecdysteroids and not JH regulate acarine vitellogenesis (Fig. 5). The evidence is clear in ticks that ecdysteroids appear in the female during the time of vitellogenesis; ecdysteroids added in organ culture or injected into ticks will increase Vg protein levels; and ecdysteroids injected into part-fed (virgin) females of *D. variabilis* still attached to their host will initiate the expression of the Vg1 and Vg2 message, increase levels of Vg in the hemolymph, and produce vitellogenic eggs with ovary weights similar to that for replete (mated) females. Based on currently available evidence, it is reasonable to assume that this model also applies to mites rather than the alternative that mites produce and utilize JH as in insects to regulate expression of the Vg genes. In the model shown in Fig 5, the male transfers a pheromone (possible multiple factors) to the female, perhaps specifically to the female genital tract during the transfer of the spermatophore as has been shown in ticks, and this is the first step in the initiation of female reproduction. This action via the brain results in the synthesis of an ecdysiotropic hormone

(EDTH) that initiates the production of ecdysteroids in the epidermis; this has been demonstrated in ticks (Roe et al., 2008; Lomas et al., 1997). Alternatively, for acarines that reproduce asexually, the process may be initiated not by the male, but the brain once the mite or tick reaches adulthood. The secreted ecdysteroid then reaches the fat body and midgut in ticks, or the midgut and ovaries in mites to trigger the synthesis and release of Vg (Fig. 5). In ticks, it has been shown by RNAi knockdown experiments, that a Vg receptor in the ovary is absolutely required for the uptake of Vg into the eggs (Mitchell et al., 1007).

8. Future directions

Although significant advancements have been made in recent years on understanding the endocrine regulation of tick development, similar studies in mites have been greatly lacking. To better understand whether ecdysteroids or JH regulate mite reproduction, it will be critical to complete more work on the molecular biology of mite storage proteins. Currently, we have only partial sequences of Vg from one mite species and no work on the other storage proteins in mites, if they even exist. Once the molecular tools are available, similar studies to those conducted with ticks can be used to determine the site of Vg synthesis in mites and to examine the role of ecdysteroids versus JH on expression of the Vg messages. One of the challenges will be the small size of mites and the difficulty of conducting ecydsteroid injections compared to ticks. Classical studies are needed to correlate changes in ecdysteroid levels to vitellogenesis in mites and to directly determine whether mites can

synthesize the common insect JHs. It appears likely that the development of mite transcriptomes will be especially valuable in working with this group along with the use of RNAi where traditional whole organism approaches are difficult because of the size of the mite.

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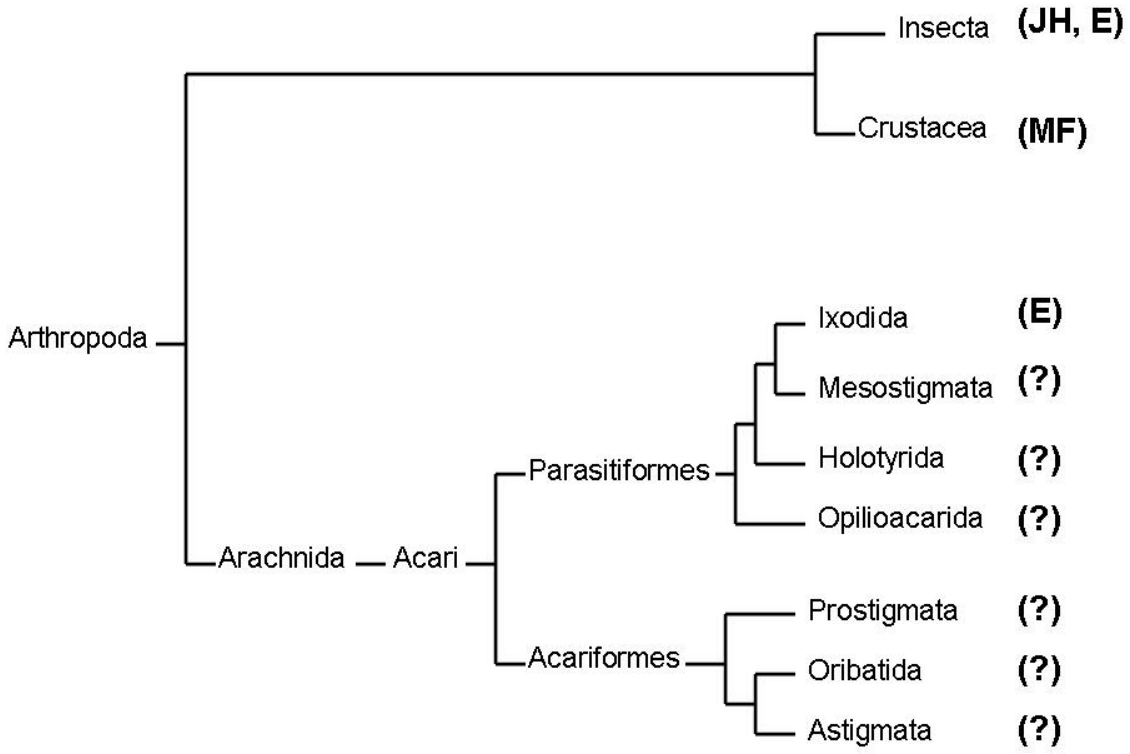

Figure 1. Known role of hormones in the regulation of vitellogenesis in the Arthropoda. E = ecdysteroids, JH = juvenile hormone, MF = methyl farnesoate, ? = unknown.

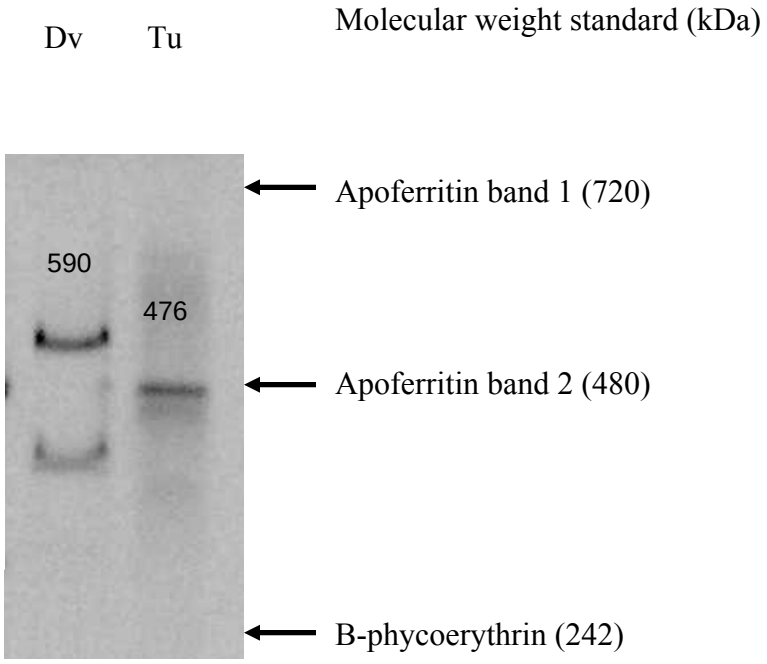

Figure 2. Clarified egg homogenate from the American dog tick, *Dermacentor variabilis* (Dv), and the twospotted spider mite, *Tetranychus urticae* (Tu) on Native PAGE (4-12% tris-glycine).The size proteins resolved is given in kDa, using NativeMark™ (Invitrogen) as molecular weight standards. The gel was stained with Coomassie Brilliant Blue R-250.

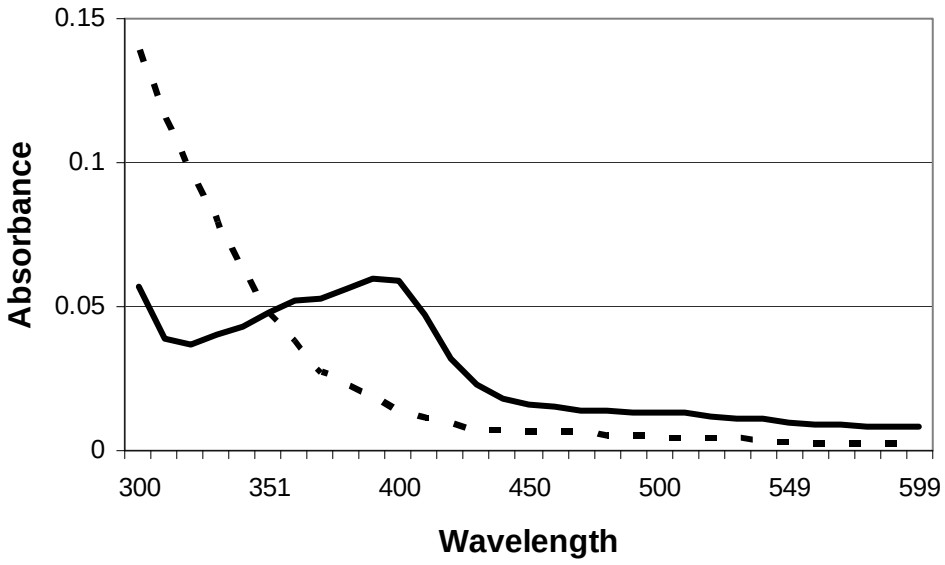

Figure 3. Light absorbance (full scan, 300-700 nm) of clarified egg homogenate from *D. variabilis* (———) versus *T. urticae* (– – –).

Table 1. Vitellogenin partial sequences (*Tetranychus urticae*) and mite ESTs (astigmatid mites) similar to arthropod and non-arthropod vitellogenin (Vg) in the NCBI database (April 2009).

Mite	NCBI accession #	ORF	# AA[a]	Top match: Vg, NCBI accession # (e-value)	Conserved motif
Prostigmata					
Tetranychus urticae	AB455063	+2	322	*Ixodes scapularis* putative Vg, EEC18889 ($1e^{-10}$)	vWD[b]
	AB455064	+2	321	*Caenorhabditis elegans*, NP_508589 ($4e^{-14}$)	vWD
	AB455065	+2	316	*Caenorhabditis elegans*, XP_001676402 ($3e^{-11}$)	vWD
	AB455066	+2	317	*Ripicephalus microplus*, ABS88989 ($2e^{-9}$)	vWD
Astigmata					
Blomia tropicalis	CB282111	+2	242	*Ripicephalus microplus*, ABS88989 ($4e^{-18}$)	vWD
	CB282841	+1	87	*Solenopsis invicta* Vg-2, AAY22960 ($4e^{-5}$)	
	CB282903	+3	132	*Athalia rosae*, BAA22791 ($9e^{-10}$)	vWD
	CB282955	+1	123	*Solenopsis invicta* Vg-3, AAY22961 ($2e^{-5}$)	
	CB283175	+3	223	*Athalia rosae*, BAA22791 ($4e^{-11}$)	vWD

Table 1. Continued.

	CB283177	+3	273	*Dermacentor variabilis* Vg-2 precursor, ABW82681 ($3e^{-7}$)	
	CB283237	+3	184	*Ripicephalus microplus*, ABS88989 ($3e^{-7}$)	vWD
	CB283245	+1	187	*Boophilus microplus* GP80 precursor, AAA92143 ($3e^{-8}$)	vWD
Dermatophagoides farinae	CB283525	+1	164	*Lethocerus deyrollei*, BAG12118 ($3e^{-6}$)	
	CB283550	+2	169	*Lethocerus deyrollei*, BAG12118 ($5e^{-7}$)	
	CB283721	+3	153	*Lethocerus deyrollei*, BAG12118 ($2e^{-7}$)	
	CB284012	+1	199	*Ixodes scapularis* putative, EEC18889 ($3e^{-10}$)	vWD
	CB284246	+1	164	*Lethocerus deyrollei*, BAG12118 ($4e^{-7}$)	
	CB284331	+1	186	*Ixodes scapularis* putative, EEC18889 ($2e^{-20}$)	vWD
	CB284380	-3	180	*Dermacentor variabilis* Vg-2 precursor, ABW82681 ($2e^{-30}$)	
	CB284561	+1	161	*Lethocerus deyrollei*, BAG12118 ($6e^{-6}$)	
	CB284677	+1	145	*Lethocerus deyrollei*, BAG12118 ($5e^{-6}$)	
	CB284726	+1	169	*Lethocerus deyrollei, BAG12118 (6e-6)*	
	CB284828	+2	177	*Solenopsis invicta Vg-2, AAY22960 (2e-7)*	

Table 1. Continued.

	CB285073 CB285075	+1 +1	138 132	*Lethocerus deyrollei*, BAG12118 ($8e^{-7}$) *Lethocerus deyrollei*, BAG12118 ($8e^{-6}$)	
Dermatophagoides pteronyssinus	EX162239	+3	173	*Ripicephalus microplus*, ABS88989 ($3e^{-9}$)	
Glycyphagus domesticus	CO436528 CO438427 CO438632	+1 +3 +1	108 108 118	*Nasonia vitripennis*, XP_001607388 ($5e^{-6}$) *Caenorhabditis elegans*, NP_509305 ($2e^{-6}$) *Pimpla nipponica*, AAC32024 ($8e^{-6}$)	
Sarcoptes scabiei	BM521995 BM522186	+2 +2	88 140	*Pimpla nipponica*, AAC32024 ($7e^{-7}$) *Pimpla nipponica*, AAC32024 ($4e^{-10}$)	 vWD
Suidasia medanensis	CN738096	+2	116	*Caenorhabditis elegans*, NP_509305 ($2e^{-6}$)	

Each sequence was submitted to the open reading frame (ORF) finder in the NCBI website (http://www.ncbi.nlm.nih.gov/). The ORF reported corresponds to the translated peptide similar to Vg. The ORF finder also provides the estimated length of the translated peptide (# AA). A comparison of the translated peptide with the NCBI protein database (blastp) showed a list of similar sequences; the top match for Vg is shown (species, NCBI accession number and e-value).

[a]AA: amino acids
[b]vWD: von Willebrand factor D

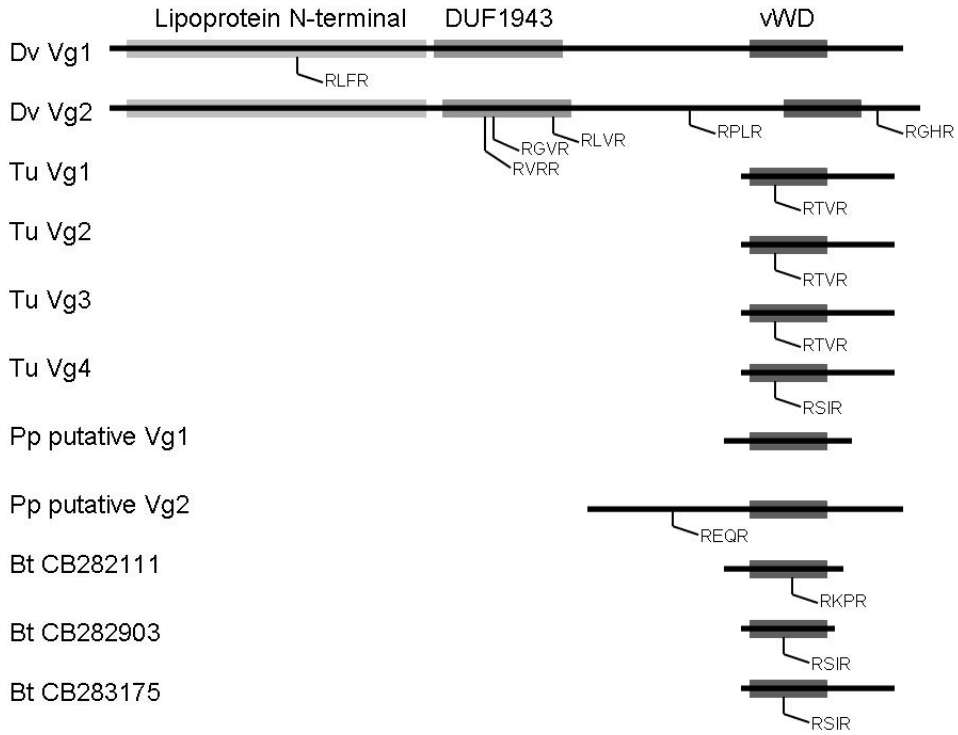

Figure 4. Graphical representation of selected translated amino acid sequences of vitellogenin from *Dermacentor variabilis* (Dv Vg1, Dv Vg2), *Tetranychus urticae* (Tu Vg1, Tu Vg2, Tu Vg3, Tu Vg4), *Phytoseiulus persimilis* (Pp putative Vg1, Pp putative Vg2) and *Blomia tropicalis* (Bt CB282111, Bt CB282903, Bt CB283175). Translation of DNA sequences to amino acid sequences were obtained with the Open Reading Frame (ORF) finder, in the NCBI website (http://www.ncbi.nlm.nih.gov/). Alignments of amino acid sequences were conducted using the program Vector NTI (Invitrogen). All present cleavage sites RXXR are included.

Table 2. The effect on mite reproduction of exogenous arthropod hormones, JH analogs, ecdysone analogs and ecdysone antagonist.

Hormone analog/antagonist	Mite species	Effect on reproduction[a]	Reference
Farnesol	*Tetranychus urticae*	+	Regev and Cone 1976
	Tyrophagus putrescentiae	0	Czaja-Topinska et al. 1979
JH III	*Dermanyssus gallinae*	+	Oliver *et al*. 1985
	Varroa jacobsoni	+	Hänel 1983
	Varroa jacobsoni	+	Hänel and Koeninger 1986
JH Analogs			
CGA 29'170	*Tetranychus urticae*	-	Klein-Koch 1976
	Phytoseiulus persimilis	-	
Fenoxycarb	*Acarus siro*	+	Thind and Edwards 1990
	Dermatophagoides farinae	-	Downing et al. 1990
Hydroprene	*Dermatophagoides farinae*	-	Downing et al. 1990, 1993

Table 2. Continued.

Methoprene	*Amblyseius brazilli*	-	El-Banhawy 1977, 1980
	Dermatophagoides farinae	-	Downing et al. 1990, 1993
	Tetranychus desertorum	-	El-Banhawy 1980
	Tyrophagus putrescentiae	0	Czaja-Topinska *et al*. 1979
Pyriproxyfen	*Tyrophagus putrescentiae*	0	Sánchez-Ramos and Castañera 2003
	Neoseiulus fallacies	0	Villanueva and Waldenbach 2005
	Stratiolaelaps scimitus	0	Cabrera et al. 2004
S-Kinoprene	*Phytoseiulus persimilis*	-	Bostanian and Akalach 2006
	Amblyseius fallacis	0	Bostanian and Akalach 2006
ZR-777	*Tetranychus urticae*	-	Klein-Koch 1976
	Phytoseiulus persimilis	-	
Ecdysone Analogs			
Halofenozide	*Tyrophagus putrescentiae*	-	Sánchez-Ramos and Castañera 2003
Methoxyfenozide	*Neoseiulus fallacis*	0	Villanueva and Walgenbach 2005

Table 2. Continued.

Ecdysone Antagonist			
Diofenolan	*Euseius addoensis*	+	Grout et al. 1997
	Euseius citri	+	

[a] + increase in oviposition, - reduction in oviposition, 0 no difference in oviposition compared to the negative control.

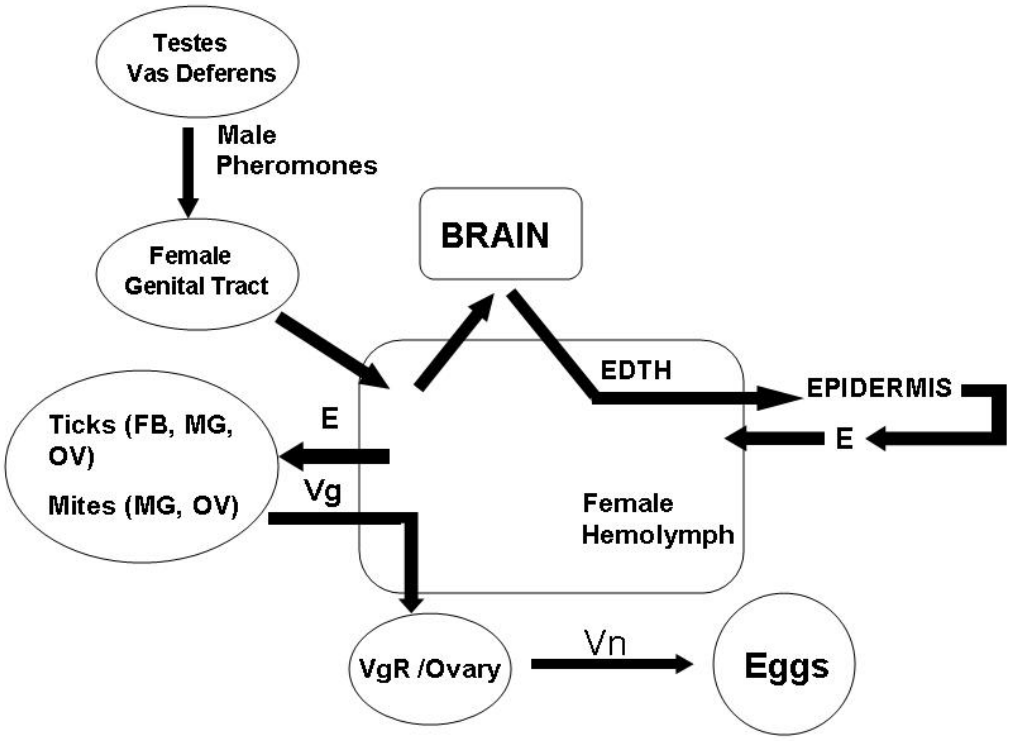

Figure 5. Proposed model for the regulation of vitellogenesis in the Acari. The transfer of male pheromones to the female stimulates the brain (synganglia) to secrete ecdysiotropic hormone (EDTH) to the hemolyph. EDTH then stimulates the epidermis to produce ecdysteroids (E), which in tissues such as fat body (ticks), midgut (ticks, mites) and ovary (ticks, mites) initiates synthesis of vitellogenin (Vg). Vg uptake into the oocytes occurs via Vg receptors (VgR) and is then called vitellin (Vn). Vitellin then serves as a nutritious source for the developing embryo in the eggs. FB: fat body, MG: midgut, OV: ovary.

Chapter 3

Characterization of vitellin protein in the twospotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae)

This work was published in 2009 in the Journal of Insect Physiology 55, 655-661. Authors Ana R. Cabrera, Kevin V. Donohue, Sayed M. S. Khalil, Daniel E. Sonenshine, R. Michael Roe.

Abstract

In mites, vitellogenin synthesis, regulation and uptake by the oocytes as vitellin remain practically unknown. Although a partial sequence of the gene is now available, no previous studies have been conducted that describe the native vitellin protein in mites. The objective of this study was to characterize vitellin in the twospotted spider mite, *Tetranychus urticae*. The native twospotted spider mite vitellin migrated as a single major band with a molecular weight of 476 ± 14.5 kDa as compared to 590 ± 25.5 kDa for vitellin from the American dog tick, *Dermacentor variabilis*. However, isoelectric focusing analysis of native spider mite vitellin showed five bands with pI values slightly acidic to neutral (pH 5.8, 6.2, 6.7, 7.0 and 7.2), as is the case for insect and tick vitellins. Reducing conditions (SDS-PAGE) also revealed multiple subunits ranging from 290.9 to 3.6 kDa and was similar to that found in *D. variabilis*. Spider mite vitellin weakly bound lipids and carbohydrates compared to the tick. Unlike *D. variabilis*, the spider mite egg yolk protein does not bind heme. The significance of non-heme binding in mites is discussed.

Key words: mite, spider mite, tick, American dog tick, vitellogenin, vitellin, yolk protein, reproduction, egg

1. Introduction

Our knowledge of the reproduction of mites and ticks in the subclass Acari is limited. The regulation of vitellogenesis has been studied in ticks to some extent, but in mites is yet not understood. Essential studies such as the characterization of the yolk protein known as vitellin, or its precursor vitellogenin (Vg), have not been performed before in any mite species.

Our best understanding of the regulation of vitellogenesis within the Acari, is with ticks. Pound and Oliver (1979) found that juvenile hormone (JH) was able to rescue the anti-JH effects of precocene 2 and initiate egg production in adult females of the soft tick, *Ornithodoros parkeri*. This study suggested that ticks were similar to insects and regulated vitellogenesis like most insects so far studied, with increases in the concentration of circulating JH. A number of pharmacology studies were conducted (recently reviewed by Roe et al., 2008) which were equivocal in determining the role for JH in female tick reproduction. However, Neese et al. (2000) were unable to detect the synthesis of any of the common insect JHs or the precursor to JH III, methyl farnesoate, in the hard tick, *Dermacentor variabilis*, or the soft tick, *O. parkeri*. In the same study, JH could not be detected in the hemolymph of these same ticks at the time of vitellogenesis by selective ion, electron impact, gas chromatography mass spectrometry or in whole body homogenates of *D. variabilis* during each stage of development by the *Galleria* bioassay. Thompson et al. (2005, 2007) sequenced the yolk protein from the American dog tick (*D. variabilis*) and were able to

show that the injection of 20-hydroxyecdysone into virgin, partfed females still attached to the host increased the level of the Vg message. These studies also showed that the yolk protein levels in the hemolymph increased, the ovaries reached the approximate size of mated replete females, and the eggs became fully developed after ecdysteroid injections. Vg was synthesized in fat body and midgut with low levels of expression in the ovary (Roe et al., 2008). These results argue that ecdysteroids and not JH regulate tick vitellogenesis, although most recently, Seixas et al. (2008) suggested other factors in addition to ecdysteroids may be important to egg maturation in *Amblyomma hebraeum*.

Similar to the studies conducted in ticks by Pound and Oliver (1979), Oliver et al. (1985) found that the application of JH could partially rescue the anti-JH effect of precocene 2 in the mesostigmatid mite, *Dermanyssus gallinae*. This led to the general view that still exists today that mites use JH to regulate egg development in a manner similar to many insects. This hypothesis has never been challenged in the mite literature, even though Czaja-Topinska et al. (1979) testing 56 JH analogs found that several of the compounds reduced egg laying, while Downing et al. (1990) found that the JH analogs methoprene and hydroprene suppressed population growth in the house dust mite, *Dermatophagoides farinae*.

No research is available describing the characteristics of yolk proteins in mites. Kawakami *et al*. (2009) recently described four partial Vg messages from *Tetranychus urticae* and showed expression was initiated at day 1 after female adult emergence. There does appear to be some differences in the role of the fat body in yolk protein synthesis

between mites and ticks. In ticks, the fat body and midgut appear to be the major source of Vg (Roe et al., 2008). Although mesostigmatid mites have fat body (Filimonova, 2001; Coons et al., 1990), the majority of species in the suborder Prostigmata do not (Filimonova, 2001; Mothes-Wagner and Seitz, 1984). In its absence, the midgut and nurse cells in the ovary may be the source of vitellin (Alberti and Crooker, 1985; Mothes-Wagner and Seitz, 1984). Histological studies on the twospotted spider mite, *T. urticae*, showed that the oocytes were associated with midgut cells (Mothes-Wagner and Seitz, 1984), while in the honey bee parasite, *Varroa jacobsoni*, the site of yolk synthesis could not be determined (Steiner et al., 1995).

The objective of the current study is to characterize the yolk protein, vitellin, in eggs of the twospotted spider mite, *T. urticae*, and compare this to tick vitellin. Such studies are a critical first step in understanding the molecular biology of these proteins, their tissue source and function, and whether the current hypothesis that JH regulates Vg synthesis in mites is correct.

2. Materials and Methods

2.1 Twospotted spider mites and American dog ticks

A twospotted spider mite, *T. urticae*, colony was established in March 2007 in greenhouses at North Carolina State University. Temperature in the greenhouse ranged from

22 to 31 ºC with natural light conditions throughout the year. The mites originated from two infested cotton plants (*Gossypium hirsutum*) provided by Michelle Meck, BASF, Research Triangle Park, NC. Common bean, *Phaseolus vulgaris* L., was used to maintain the twospotted spider mite colony; the plants were grown in 4-inch clay pots with Fafard 2 mix growing media (Conrad Fafard, Inc., Agawam, MA) and watered as needed. Mites of all stages were allowed to feed, molt and lay eggs on the leaves of 2-4 wk old plants to provide mites with an adequate food source and shelter.

American dog tick, *D. variabilis*, eggs were obtained from a pathogen-free tick colony maintained at Old Dominion University, Norfolk, Virginia. All use of animals for this research was conducted at Old Dominion University and in accordance with protocols approved by the Old Dominion University Institutional Animal Care and Use Committee. The approved protocols are on file in the Old Dominion University Animal Care Facility Office. The rearing method has been described previously (Sonenshine, 1993). American dog tick vitellin has been described before (Rosell and Coons, 1991; Thompson et al., 2005, 2007) and was used in this study as a reference since acarine vitellin has only been characterized from ticks.

2.2 Egg collection and egg homogenate preparation

Twospotted spider mite eggs were collected by placing 20 female and 2 male mites on a rectangular (~4-5 cm^2) clean piece of bean leaf (*P. vulgaris*), abaxial side up. Every 3 h,

eggs were collected using a fine paint brush, placed in ice-cold 10mM phosphate-buffered saline (PBS: 0.14M NaCl, 0.003M KCl, 0.01M Na_2HPO_4, 0.002M KH_2PO_4, pH 7.4), contained in a 1.5-ml Eppendorf tube and stored at -80 ºC until processed. A sample consisted of 2000 mite eggs collected in 30 µl of PBS, and several samples were collected to conduct this study. Eggs were macerated manually using a plastic homogenizer that fit into the Eppendorf tube holding the eggs and buffer. The homogenate was then centrifuged at 16,500 x *g* at 4 ºC for 15 min. The clarified supernatant was stored at -80 ºC until used.

Replete females of the American dog tick were allowed to lay eggs for 24 h in a glass vial. Afterward, 25 eggs were collected using a fine paint brush into 500 µl of 10mM PBS contained in a 1.5-ml Eppendorf tube. Homogenization and clarification of the sample was carried out in the same manner as for the twospotted spider mite.

2.3 Protein electrophoresis

The total protein concentration in the twospotted spider mite and American dog tick egg homogenates was estimated with the BioRad protein assay (BioRad Laboratories, Hercules, CA) using bovine serum albumin (Fraction V, Fisher Scientific, Pittsburgh, PA) to prepare standard concentrations. Absorbance values and estimation of protein concentration were obtained using a Spectra Max Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA) and MaxPro software (Software Concepts, Inc., Beaverton, OR). Total protein in clarified egg homogenate was also determined by measuring absorbance at 280

versus 260 nm (Nanodrop 1000 spectrophotometer, Thermo Scientific, Waltham, MA) according to the method of Layne (1957). The total protein levels that follow were determined by the BioRad method unless otherwise specified.

Native polyacrylamide gel electrophoresis (PAGE) was used to estimate the molecular weight of twospotted spider mite vitellin under non-reducing conditions. We used 4-12% tris-glycine polyacrylamide gel, 1X tris-glycine running buffer (25 mM tris, 250 mM glycine; pH 8.3) and NativeMark Unstained Protein Standards (Invitrogen, Carlsbad, CA) as a molecular weight standards. The twospotted spider mite and tick egg homogenates were diluted with native sample buffer (Invitrogen) to load 12 and 4 μg of total protein, respectively. The gel ran in an Invitrogen XCell SureLock™ electrophoresis device for 8 h at a constant 84 volts in an ice-cold water bath. Afterward, the gel was submerged in a prefixing solution (50% methanol, 10% acetic acid and 40% water) for 1 h and then stained overnight with Coomassie Brilliant Blue R-250 (12.5 g of Coomassie Brilliant Blue R-250 dye, 225 ml methanol, 50 ml acetic acid and 225 ml water). The gel was destained (30% methanol, 10% acetic acid and 60% water) with several washes over a period of 8 h. It was necessary to load three times as much protein (as determined by the BioRad protein assay) of *T. urticae* egg homogenate than *D. variabilis* to visualize the proteins from the former after electrophoresis with Coomassie Blue stain. A linear regression analysis to estimate the molecular weight of vitellin was conducted using standard proteins (NativeMark Unstained Protein Standard).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to determine the number of vitellin subunits in the eggs of the twospotted spider mite and to estimate their molecular weight under denaturing conditions. This analysis was conducted with 4-12% tris-glycine polyacrylamide gels, 1X SDS tris-glycine buffer (25mM Tris, 250mM glycine, 0.1% SDS; pH 8.3) and BenchMark Protein Ladder (Invitrogen, Carlsbad, CA). The twospotted spider mite and tick egg homogenates were diluted with SDS sample buffer (Invitrogen) and 10mM dithiothreitol (DTT) to load 20 and 6 µg of total protein, respectively. The samples were incubated at 85 ºC for 2 min in a PTC-100 Peltier thermal cycler (MJ Research, Ramsey, MN). The gels were run with the conditions described above. Afterward, the gel was fixed, stained and destained as described before for native gel electrophoresis. Again, it was necessary to load more than three times the amount of protein in the twospotted spider mite than the American dog tick sample in order to visualize the former. Native and SDS gels were replicated three times with three different samples.

2.4 Lipid, carbohydrate and heme binding properties

To determine if twospotted spider mite vitellin binds lipids, native PAGE was conducted as described previously. The gel was stained overnight with Sudan Black B (ACROS, New Jersey; 500 mg Sudan Black B dye, 80 ml water, 20 ml acetone and 15 ml acetic acid) and destained with a mixture of 15% acetic acid, 20% acetone and 65% water

(Maurer 1971). Native gels stained with Sudan Black B were replicated three times with three different samples.

The binding of carbohydrates by twospotted spider mite vitellin was assessed using SDS-PAGE as described previously. A negative control (soybean trypsin inhibitor) and positive control (horseradish peroxidase) provided with the Gel Code Glycoprotein Staining Kit (Pierce Protein Research Products, Rockford, IL) were included in the analysis. Briefly, after electrophoresis the gel was submerged in a 50% methanol solution for 30 min and then washed twice with a 3% acetic acid solution. The gel was immersed in an oxidizing solution for 15 min, then washed with 3% acetic acid and next exposed to the GelCode® Glycoprotein Staining Reagent for 15 min. Finally, after a 5 min immersion in the reducing solution, the gel was washed again with 3% acetic acid and glycoprotein bands were identified by their magenta color. This analysis was replicated three times with three different samples.

To determine heme binding properties, a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA) was used to conduct a continuous wavelength scan (300-700 nm) of twospotted spider mite and American dog tick egg homogenates. The protein concentration was 3.8 µg/µl and 19.8 µg/µl for the twospotted spider mite and the American dog tick sample, respectively. The continuous wavelength scan was replicated twice with two different samples.

2.5 Isoelectric point

To determine the isoelectric point (pI), 50 µg each of twospotted spider mite and American dog tick egg homogenate samples were submitted to the protein facility at Iowa State University (Ames, Iowa) for analysis. The proteins were resolved on a 7 cm immobilized pH gradient mini-gel using the IPGphor Isoelectric Focusing System (Amersham Pharmacia Biotech, Piscataway, NJ). Proteins were stained with Coomassie Brilliant Blue R-250. The pIs were estimated based on migration distance. The isoelectric point analysis was replicated two times with two different samples.

2.6 Amino acid composition analysis

The amino acid composition analysis for the yolk protein in newly laid eggs (≤3 h old) of the twospotted spider mite was conducted by the company, Alphalyse (Palo Alto, CA). The sample had a protein concentration of 0.32 µg/µl of 10mM PBS. The protein was hydrolyzed for 20 h using a solution of 6N HCl, 0.1% phenol and 0.1% thioglycolic acid at 110 ºC under argon gas at 1 atm. A Biochrom 30 Amino Acid Analyzer (Biochrom Limited, Cambridge, England) was used to conduct the ion exchange chromatography analysis according to standard procedures at Alphalyse.

3. Results

In the current study, yolk proteins were analyzed from newly oviposited eggs (≤3 h old spider mite eggs and ≤ 24 h old tick eggs) when embryo development would be minimal and the expected major protein in the egg homogenates of the spider mite, *T. urticae*, and the American dog tick, *D. variabilis*, is vitellin. In preliminary studies in which the protein concentrations of these samples were determined by BioRad reagent and the proteins resolved by native PAGE with equal protein loading for mites and ticks, the protein in the former was difficult to visualize by eye as compared to that from the tick. Therefore it was necessary to load 12 and 4 µg of protein per lane (a 3-fold difference in protein concentration), respectively, for approximately equal protein staining of vitellin. The native PAGE of the total protein stained with Coomassie Brilliant Blue R-250 resolved a single major protein in the mite and tick (Fig. 1) with a molecular weight of 476 ± 14.5 (± 1SE, n=3) and 590 ± 25.5 (± 1SE, n=3) kDa, respectively, when the proteins were compared to authentic molecular weight standards. There was a statistically significant difference in protein size for vitellin between *T. urticae* and *D. variabilis* in these studies (t = 4.4017, P= 0.0117). In addition, a minor protein band was found in the tick with a molecular weight of 385 ± 13.2 kDa (Fig. 1). When the concentration of protein before loading on the gel was determined by the method of Layne (1957) using absorbance at 280 and 260 nm, it still required a 5 fold increased level of protein for the mite versus the tick to produce approximately equivalent levels of vitellin staining in the gel.

Although a single major protein was resolved by native PAGE based on molecular weight in the American dog tick and the spider mite, multiple native isoelectric forms of vitellin were found for both (Table 1). The pI values of the *D. variabilis* vitellin were 5.95, 6.3 and 6.6 and that for *T. urticae* 5.8, 6.2, 6.7, 7.0 and 7.2.

While tick vitellin appeared as a single major band on native PAGs (Fig. 1), the protein is multimeric when resolved by SDS-PAGE (Fig. 2) as has been shown before from this lab and by others (Thompson et al., 2007; Rosell and Coons, 1991). The estimated molecular weights of the different bands ranged from 17.7 to 168.4 kDa. The predominant proteins were a single band at 113.4 kDa and two bands of similar size at 80.3 and 72.6 kDa. *T. urticae* vitellin was also multimeric (Fig. 2). The largest proteins detected were of similar size (290 and 284 kDa) with no counterpart detected in *D. variabilis*. A prominent mite band was found at 176.5 kDa which corresponded to a minor 168.4 kDa band in the tick. A prominent doublet appeared in the mite at 76.2 and 67.7 kDa which appeared to correspond with a prominent doublet in *D. variabilis* at 80.3 and 72.6 kDa. In *T. urticae*, another doublet appeared at 28.4 and 26.0 kDa which was absent from the tick. There was also a corresponding range of proteins in the mite and tick between approximately 14-18 kDa, the smaller of which was more abundant in the mite.

The amino acid composition of the spider mite egg is shown in Table 2. Vitellin had a high percentage content of Glu/Gln (14.3%), Asp/Asn (12.2%), Lys (7.9%), Leu (7.8%) and Gly (7.5%) followed in decreasing content of Ala (6.6%), Ser (6.7%), Pro (5.8%), Thr (5.3%)

and Val (5.7%). The other amino acids ranged from not detectable (Trp) to 4.6% (Arg). Cys and His levels were 0.2 and 2%, respectively.

Spider mite vitellin was examined in clarified egg homogenate to see if it carried lipid, carbohydrate and heme. In these studies, the positive control was clarified egg homogenate from the American dog tick which was characterized before as a heme-glycolipo-protein (Guderra et al., 2002b; reviewed by Roe et al., 2008). Even though the amount of protein loaded on the gels was 3- or 5-fold higher (as determined by BioRad versus the method of Layne (1957), respectively) for *T. urticae* versus *D. variabilis*, the level of binding of Sudan Black B was approximately equivalent (Fig. 4). Sudan Black B binding denotes the presence of lipids. Lipid staining was noted at 476 and 590 kDa, corresponding to the molecular weight of vitellin in the respective organisms as was shown before (Fig. 1). It was clear from SDS-PAGE, that most of the subunits of vitellin from *D. variabilis* bound the GelCode Glycoprotein staining reagent (Fig. 5). Although binding also was detected for *T. urticae*, the level of staining was minimal in comparison. The positive and negative controls results were as expected (Fig. 4).

Tick vitellin from egg homogenate on native gels appeared brown in color indicative that it contains heme (data not shown) as was shown before (Gudderra et al., 2002a). Also, vitellogenic eggs are brown in color (Thompson et al., 2005). This brown coloration was not observed for eggs, egg homogenate or during native gel electrophoresis for the spider mite in our studies (data not shown). Tick vitellin produced a characteristic peak at 405 nm (Fig. 5)

indicative of the presence of heme (Gudderra et al., 2002a) while heme absorption was lacking in *T. urticae* (Fig. 5).

4. Discussion

4.1. Native yolk protein from the twospotted spider mite

Vitellogenin (Vg) circulates in arthropod hemolymph and is the precursor of the egg yolk protein, vitellin (Vn) (Raikhel and Dhadialla 1992). Vg is selectively absorbed from the hemolymph by developing oocytes via a receptor-mediated endocytosis, and this absorbed protein, following its conversion as vitellin, serves as a nutritional source for the developing embryo. Our best understanding of the protein chemistry of yolk proteins is found in insects. Vg is generally a high molecular weight oligomeric phosphoglycolipo-protein varying in size from 210 to 600 kDa. In the Crustacea (Decapoda: Penaeida, for example), Vg and Vn are lipoglyco-carotenoproteins with molecular weights of 300-560 kDa. Less is known about the proteomics of Vg and Vn in the Acari, and the vast majority of the research is with ticks (reviewed by Roe et al., 2008; Gudderra et al., 2002b). In the soft tick, *Ornithodoros moubata* and *O. pakeri*, native vitellin was reported with a molecular weight of 600 kDa (Chinzei et al., 1983; Taylor and Chinzei, 2001). In the hard tick, *D. variabilis*, Vg determined by native PAGE and gel permeation chromatography was in the range of 320-486 kDa (Sullivan et al., 1999; Gudderra et al., 2001, 2002 a,b; Thompson et al., 2005,2007), and

Vn by native PAGE was 370-480 kDa (Rosell and Coons, 1991). In the current study, the major Vn was 590 KDa (Fig. 1) with a minor band at 385 kDa, the former certainly in the upper range of reported sizes for tick yolk proteins but larger than that reported earlier for the American dog tick. The estimation of molecular weight by native PAGE has been variable in ticks and even from the same tick species as reported from this laboratory for *D. variabilis.* Although it is difficult to understand all of the reasons for this variability, Cabrera et al. (unpublished) recently found that changes in the estimation of the molecular weight of Vn from *D. variabilis* was more associated with the commercial source and choice of molecular weight markers than the electrophoretic conditions used by Thompson et al (2005) versus those used in this current study. Gudderra et al. (2001) showed before for the heme-carrier (storage) protein (CP) in *D. variabilis* that the native size was different depending on whether the estimate was conducted by gel filtration or electrophoresis. Further studies will be needed to fully understand the native size(s) of storage proteins in the Acari. However, it was clear under the conditions of native PAGE in the current study, the twospotted spider mite Vn was smaller than that of the tick, *D. variabilis*, the former with a calculated molecular weight of 476 kDa (Fig. 1). This size for the twospotted spider mite is within the range of reported sizes for tick yolk proteins reported before.

Thompson et al. (2007) provided the first full sequence of a Vg message from ticks (*D. variabilis*). The conceptual translation of the nucleotide sequence and removal of the signal sequence from this protein yielded a predicted molecular weight of 206 kDa. It was argued by Thompson et al. (2007) that since the predicted size was approximately half that of

the actual yolk protein size resolved by PAGE or gel permeation chromatography, the native protein in ticks may exist in hemolymph circulation and in the egg as a dimer. Currently, a full length sequence of the yolk protein message from mites is not available. However, considering the large size of the native protein found in *T. urticae* in the current studies and what we know about the size of this protein and its message in ticks, it appears probable that the mite yolk protein is also oligomeric. This is also a reasonable conclusion considering that oligomeric forms of this protein occur in other arthropods (Sappington and Raikhel, 1998). Further studies will be needed to confirm this hypothesis. In *D. variabilis*, Vg1 contained a high content of Leu and Val (12.9 and 12.1%, respectively) followed in decreasing abundance of Pro, Lys, Thr, Glu, Tyr and Ser (7.9, 7.8, 7.5, 7.2 and 6.6%, respectively) (Thompson et al., 2007). The abundance of the remaining aa was 5.8% or less. In the current study with the mite *T. urticae*, Leu and Val were not predominant (Table 1). However, it appears there are multiple yolk protein messages in the twospotted spider mite (discussed in more detail later), and the composition analysis reported here represents an average of the relative abundance of each in addition to the amino acid make-up of each mite Vg.

Although native spider mite vitellin appears as a single band using PAGE and Coomassie Blue stain, isoelectric focusing (IEF) revealed that the egg homogenate from *T. urticae* had five different bands that migrated to different pHs. The observed pI values for these bands were 5.8, 6.2, 6.7, 7.0 and 7.2 (Table 2). These pI values range from slightly acidic to neutral, which is the case of most insect Vgs (Raikhel and Dhadialla, 1992). Ticks were recently found to have multiple yolk protein messages (reviewed by Roe et al., 2008; Donohue et al.,

2009). Also, Kawakami et al. (2009) found four different partial vitellogenin messages from *T. urticae*, and multiple Vgs have been found in a number of insects and other arthropods (Tsang *et al*. 2003; Roe et al., 2008; Donohue et al., 2009). These proteins also appear to share a common ancestry to the acarines (Roe et al., 2008; Donohue et al., 2009). Our IEF results suggest that the multiple Vg messages found in *T. urticae* (Kawakami et al., 2009) are being translated into protein that is found in mite eggs.

4.2 Post-translational processing of yolk protein

Sappington and Raikhel (1998) noted that most insect vitellin proteins have a single cleavage site, which generates two protein subunits. This cleavage signal, RXXR, was found in the yolk protein, Vg1, from the American dog tick (Thompson et al., 2007) and like in insects (Kunkel and Nordin, 1985; Raikhel and Dhadialla, 1992; Valle et al., 1993) produced one large and one small subunit (Thompson et al., 2007). In *D. variabilis*, the predicted molecular weights after this cleavage should produce proteins of 49.5 and 157 kDa; proteins of this approximate size were resolved by SDS-PAGE by Thompson et al. (2007) and also in the current study (35.0-56.8 and 168.4 KDa, respectively; Fig. 2). There were corresponding similar bands in *T. urticae* at 176.5 and 43.4 kDa (Fig. 2). Kawakami *et al*. (2009) reported the putative amino acid sequences of four different Vg partial genes containing 320-326 amino acids. In these sequences they identified two conserved motifs commonly found in Vg genes, the von Willebrand factor D (vWD) domain and the GL/ICG motif. In addition, within

the reported vWD domain an RXXR cleavage site was observed (RTVR for Vg1-Vg3 and RSIR for Vg4). The results for *T. urticae* (Fig. 2) suggest that this cleavage site produced the expected post-translational products. The N-terminal sequence of these relevant protein fragments are needed to confirm this hypothesis.

As was the case for tick Vg and Vn resolved by SDS-PAGE (reviewed by Thompson et al., 2007 and Roe et al., 2008), there was additional possible post-translational processing of the Vg protein producing a number of additional bands. This was also the case in the current study for the American dog tick Vn (Fig. 2). Other known dibasic endoprotease recognition motifs of the sequence R/K X R/K R in addition to RXXR (Mathews et al., 1994) were not found in the Dv Vg, while eight KXXK sites homologous to KFKKAN found in Vg of the bean bug, *Riptortus clavatus* (Hirai et al., 1998) were present but not informative. In addition, Sappington and Raikhel (1998) suggested that some of the yolk protein subunits resolved by SDS-PAGE may be preparation artifacts. The twospotted spider mite Vn was even more complex than that of the American dog tick (Fig. 2). This increased complexity may be the result of additional unique protein messages found in *T. urticae* (four genes) as compared to only two identified so far in *D. variabilis*. It is interesting that there were protein-band doublets in the tick (80.3/72.5 and 17.1/14.1; Fig. 2) as was reported before for this species (Thompson et al., 2007) and examples of the same in the mite (290.9/284.4; 76.2/67.7; 28.4/26.0; Fig. 2). The mite also had four similar bands at 18.0, 17.5, 15.5 and 13.7 kDa (Fig. 2). Thompson et al. (2007) argued that the doublets in *D. variabilis* were evidence of at least two different Vg proteins and Vg messages.

4.3 Lipid and carbohydrate binding

Twospotted spider mite vitellin binds lipids and carbohydrates (Figs. 3 and 4, respectively). However, it should be noted than it was necessary to load 3 (as determined by BioRad reagent) or 5 (as determined by the method of Layne, 1957) times more protein from *T. urticae* egg homogenate as compared to that from *D. variabilis* to obtain approximate equivalent protein staining on gels (Fig. 1 and 2) and for lipid binding assays (Fig. 3). The composition analysis of mite vitellin indicates an average tyrosine abundance of 2.5%, which is 2.88-fold less than *D. variabilis* Vg1 which consists of 7.5% tyrosine residues (Thompson et al. 2007). The nearly 3-fold lower abundance of tyrosine residues in mite vitellin may explain the difference between the protein concentration measured by dye-binding and absorbance-based methods versus that observed on native and SDS-PAGs. The lipid and carbohydrate content in tick Vg is 5.5-8.5% and 4.5%, respectively (Boctor and Kamel, 1976). Because of the small size of *T. urticae* adults and the eggs, it was not possible to do this same analysis. However, it appears from gel staining that the lipid concentration in the twospotted spider mite might be less than that for *D. variabilis*, and the reduction is even more for carbohydrate.

4.4. Role of mite Vg in heme sequestration and storage

The role of yolk proteins in the sequestration and storage of heme was recently reviewed by this lab (Roe et al., 2008; Donohue et al., 2009). Since ticks are obligate blood

feeders and excess heme can be toxic (Oliveira et al. 1999), they produce unique proteins to sequester heme namely the hemeglycolipo-carrier protein (CP) and the yolk protein. In addition, since ticks so far studied have lost the ability to synthesize heme (Braz et al. 1999) and also undergo extended periods without feeding, they must have a mechanism to store heme, which is essential for cellular function. CP is the major protein found in both male and female ticks during larval, nymphal and adult development that is involved in heme binding; and there is some evidence of a similar protein in the black widow spider (Donohue et al., 2009). Once the female tick undergoes vitellogenesis, the yolk protein appears to be involved in the transport of heme to the eggs for later use by developing embryos (Donohue et al., 2008, 2009). Whether this function for Vg in mites occurs has not been previously studied. It appears that the Vg in the twospotted spider mite does not bind heme as compared to that of the American dog tick, since a typical heme absorption spectrum was absent in the former (Fig. 5). Since the twospotted spider mite is not a blood feeder, this is not surprising. Whether these mites have the ability to synthesize heme *de novo* is unknown.

In summary, vitellin in the twospotted spider mite, at the protein level was similar to that of the American dog tick. It was comprised mostly of a single, high molecular weight, oligomeric protein as determined by native PAGE that was resolved by IEF into multiple bands. The mite protein appears to also be typical of yolk proteins in general within the Arthropoda in having an RXXR cleavage site that results in the production of a large and small post-translational protein product as well as a number of other possible degradation products. Mite Vn binds lipids and carbohydrates (but not heme), and the studies conducted

suggest the relative levels of lipids and carbohydrates compared to Vn protein are less in the spider mite as compared to that found in the American dog tick.

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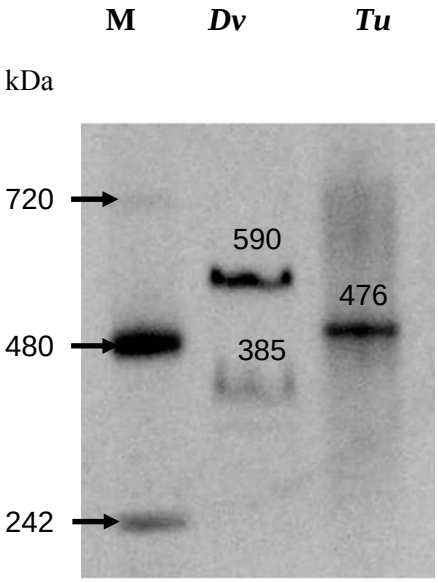
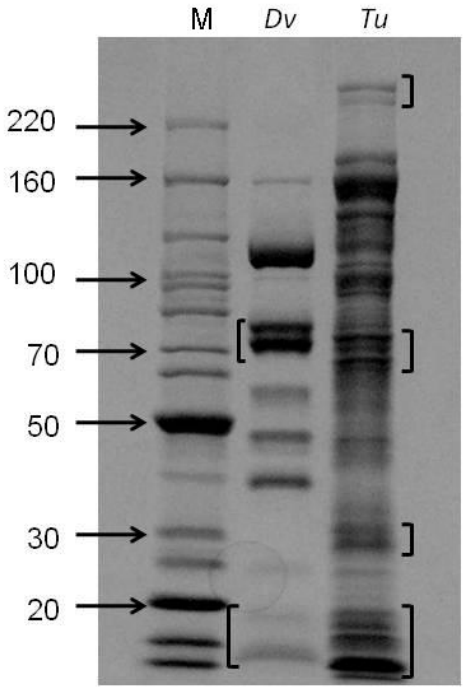


Figure 1. Native PAGE (4-12% tris-glycine) of clarified egg homogenate from the American dog tick, *Dermacentor variabilis* (*Dv*), and the twospotted spider mite, *Tetranychus urticae* (*Tu*). The size of the major protein resolved is given in kDa. The gel was stained with Coomassie Brilliant Blue R-250. M, molecular weight standards.



Molecular Weight (kDa) ± SE	
D. variabilis	*T. urticae*
	290.9 ± 14, 284.4 ± 12
168.4 ± 6.1	176.5 ± 2.1
113.4 ± 3.5	125.1 ± 3.4
80.3 ± 2.5, 72.6 ± 1.4	76.2 ± 0.7, 67.7 ± 0.8
56.8 ± 1.3 43.9 ± 0.4 35.0 ± 0.4	43.4 ± 0.6
	28.4 ± 0.6, 26.0 ± 0.3
17.7 ± 0.4, 14.1± 0.3	18.0 ± 0.2, 17.5 ± 0.2, 15.5 ± 0.4, 13.7 ± 0.4

Figure 2. Left, SDS PAGE (4-12% tris-glycine) of clarified egg homogenate from *D. variabilis* (*Dv*) and *T. urticae* (*Tu*) with groups of some bands highlighted with brackets. The corresponding molecular weights are shown on the right. The gel was stained with Coomassie Brilliant Blue R-250. M, molecular weight standards.

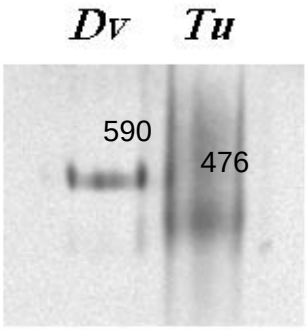


Figure 3. Native PAGE (4-12% tris-glycine) of clarified egg homogenate for *D. variabilis* (Dv) and *T. urticae* (Tu). The gel was stained with Sudan Black B to detect lipids. The size of the major band detected is in kDa.

STI HP *Dv* *Tu*

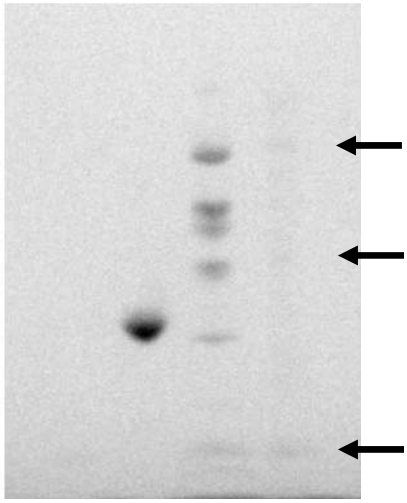


Figure 4. SDS PAGE (4-12% tris-glycine) of clarified egg homogenate from *D. variabilis* (Dv) and *T. urticae* (Tu). The gel was stained using the GelCode® Glycoprotein Staining reagent to detect glycoproteins. The negative control was soybean trypsin inhibitor (STI). The positive control was horseradish peroxidase (HP). Even though three fold more protein was resolved for Tu versus Dv, proteins in the former were hard to detect and were marked with arrows.

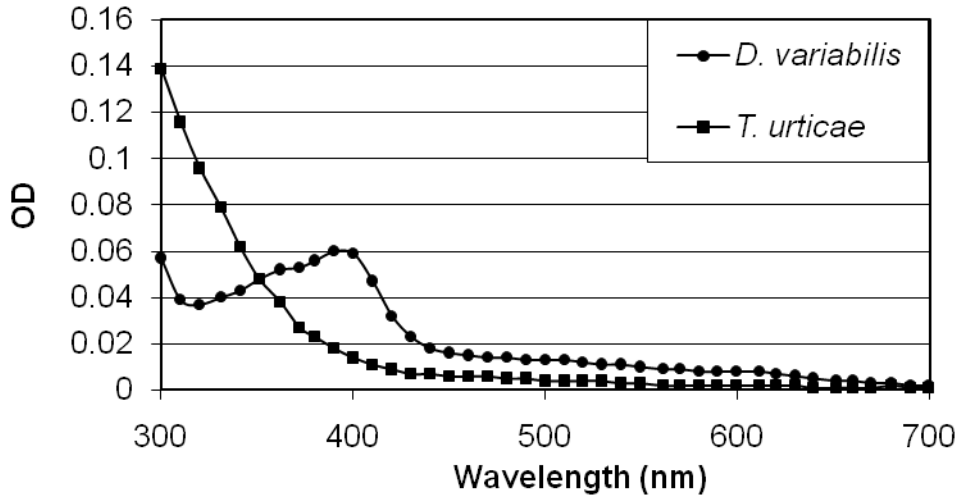


Figure 5. Absorption spectrum of clarified egg homogenate from *D. variabilis* versus *T. urticae*.

Table 1. Isoelectric points of native proteins in clarified egg homogenate from *D. variabilis* versus *T. urticae*.

D. variabilis	*T. urticae*
5.95	5.8
6.3	6.2
6.6	6.7
	7.0
	7.2

Table 2. Amino acid composition of clarified egg homogenate from *T.* urticae. Values represent molecular percentage (%).

Amino Acid	*T. urticae* (%)
Ala	6.6
Arg	4.6
Asp/Asn	12.2
Cys	0.2
Glu/Gln	14.3
Gly	7.5
His	2
Ile	5
Leu	7.8
Lys	7.9
Met	2.5
Phe	3.5
Pro	5.8
Ser	6.7
Thr	5.3
Trp	0
Tyr	2.5
Val	5.7
Total	100

Chapter 4

New approach for the study of mite reproduction: the first transcriptome analysis of a mite, *Phytoseiulus persimilis* (Acari: Phytoseiidae)

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Abstract

Several species of mites and ticks are of agricultural and medical importance. Much can be learned from the study of transcriptomes of mites, which can generate DNA-sequence information of potential target genes. High throughput transcriptome sequencing can also yield sequences of genes critical during physiological processes poorly understood in acarines i.e. the regulation of female reproduction. The predatory mite, *Phytoseiulus persimilis*, was selected as a model organism to conduct a transcriptome analysis with the 454 pyrosequencing technology. The objective of this project was to obtain DNA-sequence information of expressed genes from *P. persimilis*, with special interest in sequences corresponding to vitellogenin (Vg) and vitellogenin receptor (VgR) genes. These genes are critical to understanding vitellogenesis, and they will facilitate the study of the regulation of female reproduction in mites. A total of 12,556 contiguous sequences (contigs) were obtained, with an average size of 935 bp. From those, the putative translated peptide of 11 contigs were similar in amino acid sequences to other arthropod Vgs, while 6 were similar to VgRs. Six Vg and 3 VgR contigs were selected to conduct stage-specific expression studies. Two Vg contigs (11791 and 12365) are highly likely to correspond to two different *P. persimilis* Vgs.

Introduction

The Acari is an important group of arthropods that includes medical, livestock and agricultural pests, such as ticks and spider mites (Klompen et al., 1996; Huffaker et al., 1969). The biology, ecology and behavior of several species of ticks and mites have been extensively studied, but studies at the genomic and transcriptomic levels are scarce. Recently, the genome of the deer tick *Ixodes scapularis* was sequenced and is now available at the VectorBase website (www.vectorbase.org). Although much can be learned about the acarines by the study of the *I. scapularis* genome, ticks represent one group (suborder Ixodida) of the Acari and there are great differences between tick and mite genomes. For example, the size of the deer tick genome is close to 1.7 Gbp in size (www.vectorbase.org), while mite genomes range between 75-90 Mbp (Hoy 2009). There is intention in the near future to sequence the genome of the predatory mite *Metaseiulus occidentalis* (Hoy, 2009) and we are aware of a genome sequence project for the twospotted spider mite, *Tetranychus urticae*, but these resources are not currently available.

The predatory mite, *Phytoseiulus persimilis*, belongs to the order Parasitiformes and is closely related to ticks (Krantz, 1978). Phytoseiid mites are mostly found in the foliage of herbaceous plants and trees where they feed on all life stages of their prey (McMurtry and Croft, 1997). This mite is readily available and commercialized as a biological control agent for spider mites (Cote et al., 2002). The regulation of female reproduction in this species, and mites in general, has not been studied in depth (Cabrera et al., 2009). The prevailing

assumption that mites and ticks regulate their reproduction similarly to insects, with increasing levels of juvenile hormone (JH), may not be correct. Cabrera et al. (2009) recently reviewed the literature and proposed a new model where ecdysteroids, and not JH, are expected to regulate vitellogenesis in mites. Obtaining DNA-sequence information of vitellogenin (Vg) and vitellogenin receptor (VgR) genes will help to advance the study of the regulation of female reproduction in mites. As a result, we selected *P. persimilis* to conduct a 454 transcriptome pyrosequencing project, because this species could be used as a model organism for studies of mite reproduction at the molecular level. To our knowledge, this is the first effort to sequence a transcriptome of a mite with a high-throughput technology like 454. A recent search in Genbank (January 2010, www. ncbi.nlm.nih.gov) showed that there are currently 97 gene and 26 protein sequences of *P. persimilis*, none of them Vg or VgR. Therefore, the objective of this study was to generate DNA-sequence information of expressed genes from the predatory mite *P. persimilis* and obtain partial or complete sequences of Vg and VgR genes that can be used in the future to study the regulation of female reproduction in mites.

Materials and Methods

Mites

Predatory mites, *Phytoseiulus persimilis* (Acari: Phytoseiidae), were purchased from IPM Laboratories, Inc. (New York). Mobile stages including larva, protonymph, deutonymph, and adult male and female, were collected in a 1.5-ml Eppendorf tube and weighed. The majority of collected mites were inmmature stages, followed by female adults and very few males. A sample consisted of approximately 1 – 1.5 mg of all mobile mite stages. Immediately afterward, 50 µl of TRI Reagent (Sigma-Aldrich, St. Louis, MO) were added to each sample and the mites homogenized using a Fisherbrand disposable pestle system (Fisher Scientific, Pittsburgh, PA). The samples were stored at -80 °C until the construction of the cDNA library.

Construction of a mite cDNA library for 454 pyrosequencing

Six samples with mites previously homogenized in TRI reagent were combined to prepare a cDNA library; all mites combined weighed 7 mg. The procedure for the construction of the mite cDNA library was based on the library preparation protocol described by Donohue et al. (2010). Following the TRI reagent protocol for total RNA extraction, we obtained approximately 80.6 µg of total RNA collected in 50 µl of 100 µM aurintricarboxylic acid (ATA), known to prevent RNA degradation (Hallick et al., 1977).

Then, the Oligotex mRNA isolation kit (Qiagen, Valencia, CA) was used to isolate mRNA, according to the manufacturer's recommendations. The mRNA was precipitated with 3M potassium acetate and ethanol, and re-suspended in 2 µl of RNAse free water.

First strand cDNA synthesis was set with the mRNA combined with 10 pmol of modified 3' reverse transcription primer (5'-ATTCTAGAGACCGAGGCGGCCGACATGT(4)GT(9)CT(10)VN-3') (Beldane et al., 2006) and 10 pmol of SMART IV oligo (5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG-3') (Zhu et al., 2001), and heated at 72 °C for 2 min. Afterward, the tube containing mRNA and primers was placed immediately on ice and the following reagents were added: 2 µl of 5X first-strand buffer, 1 µl of 20mM dithiothreitol (DTT), 1 µl dNTP mix (10mM each), 1 µl RNAse Out (40 U/µl) and 1 µl of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA), and incubated at 42 °C for 90 min. The reaction was diluted to 30 µl with 1X TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.5), heated at 70 °C for 15 min, and then stored at -80 °C until used.

Prior to the second strand cDNA synthesis, the number of PCR cycles was optimized. A sample of 5 µl of first strand cDNA was used as DNA-template and combined with 10 pmol of modified 3' PCR primer (5'-ATTCTAGAGGCCGAGGCGGCCGACATGT(4)GTCT(4)GTTCTGT(3)CT(4)VN-3') (Bendale et al., 2006), 10 p mol of 5' PCR primer (5'-AAGCAGTGGTATCAACGCAGAGT-3') (Zhu et al., 2001), 5 µl 10X reaction buffer, 2 µl

MgSO4, 1 µl dNTP mix, 0.4 µl Platinum HiFi Taq Polymerase (Invitrogen) and 34.6 µl water. The thermal cycler (PTC-100™ Peltier Thermal Cycler, MJ Research, Waltham, MA) was programmed with the following conditions: 94 °C for 2 min, followed by 25 cycles with 94 °C for 20 sec, 65 °C for 20 sec and 68 °C for 6 min. Aliquots of 5 µl from cycles 12, 15, 18, 22 and 25 were taken and evaluated with a 1% agarose gel. We selected 25 cycles and ran 5 additional reactions to generate enough cDNA for sequencing, with the same thermal cycler program previously described. The cDNA was purified from each reaction using a PCR purification kit (Quiagen) according to the manufacturer's recommendations. The samples were combined and the concentration measured using a nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). Five µg of purified cDNA were submitted to the Genome Science Laboratory at North Carolina State University for pyrosequencing with the 454 technology. The cDNA library was further prepared for pyrosequencing on the GS-FLX sequencer with titanium reagents (Roche, Indianapolis, IN), with the proper kits (Roche) according to manufacturer's recommendations as described by Margulies et al. (2005).

Bioinformatics analysis

The processing of the obtained DNA sequences was carried out as described by Donohue et al. (2010). The GS Assembler ver. 1.1.02.15 (Roche) removed the primer sequence contamination and assembled sequencing reads with the following default

parameters: seed step: 12, seed length: 16, seed count: 1, minimum overlap length: 40, minimum overlap identity: 90%, alignment identity score: 2, and alignment difference score: -3. Assembled contiguous sequences, referred to as contigs, were identified using the Tera-BLASTX algorithm with DeCypher (TimeLogic) against the non-redundant (nr) and expressed sequence tag (est) databases in Genbank (downloaded February 2009). Contigs were also compared to the *Ixodes scapularis* genome available at the VectorBase website (www.vectorbase.org). The gene ontology (GO) analysis was conducted using the program Blast2GO (www.blast2go.org, Valencia, Spain). Individual contigs with at least one match similar to vitellogenin (Vg) and vitellogenin receptor (VgR) were further studied using the Open Reading Frame tool in Genbank to retrieve more information about the putative translated peptides. ClustalW (www.ebi.ac.uk/clustalw) was used to perform sequence alignments.

Stage-specific expression studies

Six contigs with similitude to arthropod Vgs and three to arthropod VgRs were selected for stage-specific expression studies. These contigs were selected based on one or more of the following criteria: counts, presence of conserved motifs and length. The selected putative Vg contigs were 00558, 08149, 10299, 11791, 11824 and 12365 and the selected putative VgR contigs were 00618, 08209 and 09459. A contig similar to lysozyme (contig 05664) was included as a non-stage specific control. Primers were designed for each contig

using VectorNTI (Invitrogen) with the following settings: minimum CG content: 50%, melting temperature (Tm) range: 50-70 °C; Table 1 contains the list of primers and their sequences.

Total RNA was extracted from three different samples (mites) of the different *P. persimilis* life stages including egg, larva, protonymph, deutonymph, and adult male and female, using a PicoPure RNA extraction kit (Molecular Devices, Sunnyvale, CA) according to manufacturer's recommendations. For each sample, cDNA was synthesized by combining 50 ng of total RNA with 10 pmol of odtankr primer (5'-CCCACAGGCACTACGATGTATTTTTTTTTTTTTTTV-3'), 1 µl dNTP mix (10mM each) and water to 12 µl before incubating at 65 °C for 5 min**.** This step was followed by the addition of 4 µl of 5X first-strand buffer, 2 µl of 100mM DTT and 1 µl RNAse Out (40 U/µl) (Invitrogen) and a second incubation at 42°C for 2 min**.** One last component was added to the reaction, 1 µl of Superscript II reverse transcriptase (Invitrogen), followed by incubation of the reaction at 42 °C for 50 min; afterward, the reaction was heated at 70 °C for 15 min.

The presence of mRNA corresponding to each DNA-sequence (contig) was evaluated with the primers, which were designed to amplify a fragment between 140 and 256 bp long. These reactions contained 2 µl of cDNA, 10 pmol of forward and reverse primers corresponding to a specific contig (see Table 1), 8.5 µl of water, and 12.5 µl of Green Master Mix (Promega, Madison, WI). The thermal cycler was set with the following program: 95 °C for 2 min, followed by 35 cycles with 95 °C for 30 sec, 30 sec with the corresponding

annealing temperature (Table 1) and 72 °C for 1 min, with a final cycle of 72 °C for 5 min. A reaction with no cDNA was included as a negative control. Presence or absence of the amplified fragments was evaluated for each contig with each life stage and the control in a 1% agarose gel as shown in Figure 3. This process was repeated 3 times for each contig and each life stage.

Results

An overview of the transcriptome of Phytoseiulus persimilis

The total number of reads from the *P. persimilis* transcriptome obtained with 454 pyrosequencing was 517,000. The GS Assembler generated 12,556 contigs ranging from 100 to 5,790 bp, with an average length of 935 bp, although the majority of contigs were 350-400 bp long. The longest contig (01475) with 5,790 bp had no similarity to any other sequence in Genbank. Comparisons of contigs with the non-redundant and EST databases in Genbank with BLASTx showed 9,598 contigs had at least one match with an e-value cutoff of 10 (76.4% of expressed genes); from those, 4,862 contigs had at least one match with a e-value cutoff of 1e-05 (38.7% of expressed genes). Novel contigs with no similar sequences to others in Genbank were 2,958 (23.6% of expressed genes). Table 2 contains the 50 most abundant contigs obtained from the *P. persimilis* transcriptome. Twenty one out of the 50 contigs (42%) were classified as unknown, with no similar sequence found in Genbank. The

putative Vg contig 11791 was one of the most abundant expressed genes with 371 reads (Table 2).

A vast number of contigs had similar sequences (BLAST hits; 4,862 contigs with e-value ≤ 1e-05) to gene sequences of non-arthropod and arthropod model organisms (Figure 1). Sixteen out of 28 of these model organisms were arthropods. The *P. persimilis* transcriptome had a large number of sequences similar to the deer tick *I. scapularis*, with almost 2,500 matches; both *P. persimilis* and *I. scapularis* belong to the order Parasitiformes, subclass Acari. Contigs categorized as cellular components included genes found in the nucleus (212 expressed genes), cytoplasm (244 expressed genes), membranes (224 expressed genes), and mitochondria (101 expressed genes). Several contigs with putative molecular function were classified into several groups, the majority with binding properties to nucleic acid (484 expressed genes), protein (336 expressed genes) and ions (228 expressed genes). Forty contigs were categorized as transcription factors and 34 contigs involved in translation initiation. Contigs with putative biological processes included genes that regulate transcription (72 expressed genes), are involved in proteolysis (60 expressed genes), translation (43 expressed genes), metabolic processes (42 expressed genes), and electron transportation (40 expressed genes). A distribution of 630 contigs within major categories of biological processes at level 2 GO analysis is shown in Figure 2.

Searching for genes involved in reproduction: Vitellogenin (Vg) and vitellogenin receptor (VgR)

Contigs with at least one match to Vg and VgR with an e-value of 5 were selected for further studies, using the Open Reading Frame (ORF) tool in Genbank and alignments with ortholog sequences. Other contigs in the *P. persimilis* transcriptome were not studied. Using the ORF tool to compare the translated peptide to a protein database in Genebank with the BLASTp algorithm, we observed similitude to arthropod Vgs in 11 contigs (Table 3). In turn, 6 putative VgR contigs were similar to low density lipoprotein receptors or VgRs of arthropods (Table 4). The top matches to these contigs had an e-value of 1e-05 or lower (Tables 3 and 4).

Characteristic conserved motifs for Vgs were observed in translated peptides from contigs 00558, 03748, 11478, 11791 and 12365 (Table 3). Contig 03748 had the Vitellogenin_N domain and the remaining contigs had the Von Willebrand factor D (vWD) motif. None of the contigs had the motif of unknown function DUF1943, also present in Vgs (Donohue et al., 2009). In turn, VgR conserved motifs were observed in some *P. persimilis* contigs including LDL_receptor_b (low density lipoprotein receptor; contigs 03434, 06415 and 07393), LDLa (contig 8209) and EGF_CA (epidermal growth factor; contig 09459) domains.

Putative Vgs and VgRs

Expression studies with *P. persimilis* contigs similar to arthropod Vgs showed that the six expressed genes evaluated were present in female samples (Table 5). The mRNA corresponding to contig 00558 was also present in all the other life stages, although not in every replicate (egg: 2, larva: 3, protonymph: 2, deutonymph: 3 and male: 1 replicate) and the brightness of the fragment was faded, possibly indicating low levels of expression. Similar results were observed for contigs 08149, 10299 and 11791, although no presence of the message was observed in any of the male samples (Table 5). The transcripts with corresponding sequences to contigs 11824 and 12365 were present in two of the three egg samples, at low levels. This was also the case for the 3 contigs with similitude to VgRs (00618, 08209 and 09459), with transcripts expressed in the female (intense brightness) and egg (faded brightness) samples. The message for putative lysozyme used as a control was present in all samples at relatively the same levels, except in the egg sample where it was not present (Table 5).

We conducted aligments with translated peptides from putative Vg contigs 11791 and 12365 because these contigs were the longest sequences (863 and 2310 bp, respectively) with a conserved motif. These sequences were compared to the complete Vg protein sequences of two tick species, *Dermacentor variabilis* and *Ixodes scapularis*; alignment with two spotted spider mite Vg were not conducted because only partial gene sequences are available. Alignments of contigs 11791 and 12365 with *D. variabilis* and *I. scapularis* Vgs showed that

out of 240 residues, these *P. persimilis* expressed genes had 47 residues at the same positions (Figure 4). Moreover, they had the characteristic Vg sequence CL/ICG, which also aligned at the same location with the tick Vgs (Figure 4). The alignments also showed that towards the C-terminus of the translated peptides there were some conserved residues in Vg sequences including glutamic acid (E), proline (P), tyrosine (Y) and aspartic acid (D). Alignments of translated peptides from all putative VgR with *D. variabilis* and *I. scapularis* VgR amino acid sequences were not informative (data not shown).

Discussion

Exploring mite transcriptomics

To our knowledge, this is the first transcriptome analysis of a mite and it can bring an insight into the research area of mite genomics. With an estimated 75-90 Mb (Hoy 2009), mites have some of the smallest genomes in the animal kingdom. The genome of *Phytoseiulus persimilis* is arranged in 4 chromosomes (2n=8) (Oliver, 1977). We obtained 12,556 contigs corresponding to potentially the same number of expressed genes from *P. persimilis* mobile stages. Previously, partial and complete gene sequences from *P. persimilis* available at Genbank totaled 97, thus our project represents a major contribution to the knowledge of DNA-sequences of expressed genes of this mite species. More than a fifth of the obtained contigs were novel sequences and from the 50 most abundant contigs, 21 were of unknown function. These results show our understanding of mite genes is in its infancy

and more work is necessary to characterize genes and determine their function in the Acari.

A large portion of the obtained sequences were similar to sequences in other arthropods as shown in Figure 1. These included several species of insects and the deer tick, *Ixodes scapularis*; the latter is currently the closest related model organism with an available sequenced genome. There were also a great number of similar sequences of *P. persimilis* transcripts to gene sequences of some non-arthropod model organism including primates, a frog, a mouse and a bird. These sequences may correspond to expressed genes with basic functions in eukaryotic cells such as ribosomal proteins, proteasomes, transcription, translation elongation and splicing factors, and others.

An insight into the reproduction of mites

Our understanding of the reproduction of mites at the molecular level is limited. Identifying and characterizing Vg and VgR genes from *P. persimilis* will facilitate the study of the site of synthesis of these genes and the hormonal regulation of vitellogenesis in the Acari; the latter represents a new area that can be exploited for finding new ways of mite pest control. Vg is the major yolk protein serving as a food source for the developing embryo (Tufail and Takeda, 2008). Vg in insects is synthesized in the fat body and cell follicles, while in ticks the synthesis occurs in the fat body, midgut and ovaries (Thompson et al., 2007); the site of synthesis in mites has not been determined yet (Cabrera et al., 2009). Vg uptake by the oocytes occurs through a VgR mediated pynocytosis (Mitchell et al., 2007).

The Vg genes of vertebrates and invertebrates share several characteristics. The length of these genes is generally 6-7 kb (Tufail and Takeda, 2008). The size of the Vg proteins is close to 2000 residues and Vgs are characterized by the presence of three conserved motifs including an N-terminal, DUF1943 and von Willebrand factor D (vWD) motifs (Donohue et al., 2009). The N-terminal (Vitellogenin_N) motif is found close to the N-terminal region and is a lipid binding region (Anderson et al., 1998), the function of the DUF1943 motif is unknown (Donohue et al., 2009) and the vWD motif interacts with the VgR, thus facilitating the uptake of Vg into the oocytes (Kawakami et al., 2009). Other conserved amino acid sequences found in Vgs include the CL/ICG and RXXR cleavage sites. The CL/ICG site is found towards the C-terminus of the peptide and its roles is essential for the oligomerization of the Vg protein (Tufail and Takeda, 2008). One cleavage site with the residue sequence RXXR is generally found towards the N-terminus region in most insect and tick Vgs (Thompson et al., 2007), but there are some insects with multiple RXXR sites distributed throughout the gene (Tufail and Takeda, 2008). Three transcripts with translated peptides similar to Vgs (contigs 00558, 11791 and 12365) had the vWD and CL/ICG motifs present; transcripts 11791 and 12365 were found in female and some immature samples but not in the male samples. To the contrary, transcript 00558 was found in a sample from a *P. persimilis* male. Vg is primarily a female-specific expressed gene, but there are some accounts of Vg expression in immature and male insects (Tufail and Takeda, 2008). Thus, transcripts 11791 and 12365 are likely to be *P. persimilis* Vgs, but with the available information, we cannot assert if transcript 00558 is also a Vg. More information is required,

including obtaining more or complete sequences of these transcripts, as well as determining the size of the mRNAs to assign function to these expressed genes.

VgR genes belong to the low-density lipoprotein receptor (LDLR) superfamily (Mitchell et al., 2007). The size of VgR genes is comparable to the size of Vg genes; for example, VgR from the American dog tick, *D. variabilis*, is almost 6 kb in length and is translated into a peptide of almost 1800 amino acids (Mitchell et al., 2007). Characteristic conserved amino acid sequences in VgRs include several YWXD repeats, two epidermal growth factor (EGF)-like motifs, O-linked sugar, transmembrane and cytoplasmic domains (Mitchell et al., 2007). The YWDX repeats are important to maintain protein structure (Mitchell et al., 2007), while the function of the EGF-like domain is to bind calcium, which aids in protein-protein interactions and also stabilizes protein structure (Rao et al., 1995). The O-linked sugar domain is involved in the protection of the VgR from proteases, which maintains the receptor stable in the cell membrane (Magrané et al., 1999). Six *P. persimilis* transcripts were found to be similar to VgRs and LDLRs. From those, contigs 00618, 08209 and 09459 were evaluated for expression in the different mite life stages and were found only in female and egg samples. The translated peptide from transcript 08209 contains a low density lipoprotein receptor (a LDLa domain), while the peptide corresponding to transcript 09459 has an EGF-CA domain. At present, there are only short sequences available from these expressed genes (176-618 bp), which prevents determination of their putative function. It may be possible that the sequences available for these three transcripts belong to the same gene. Thus, more information is needed to determine if they are indeed *P. persimilis* VgR.

Multiple Vgs in the Acari

Recently Kawakami et al., (2009) reported four different partial Vg sequences from the twospotted spider mite, *Tetranychus urticae*. This was the first account of Vg in mites at the gene level thus far. There are two known Vg genes from the American dog tick, *D. variabilis* (Thompson et al., 2007; Roe, Khalil and Sonenshine, personal communication) and three from *Haemaphysalis longicornis*, another ixodid tick (Boldbaatar et al., 2010). Sequence information available from the deer tick *I. scapularis* genome indicates there are two putative Vgs in this species (Accession No. XM_002415179.1 and XM_002403922.1). Results from the transcriptome analysis of *P. persimilis* show this predatory mite expresses at least two Vg genes.

The presence and expression of multiple Vg genes also occurs in other taxa including insects, crustaceans, and vertebrates. For instance, multiple Vgs have been cloned and characterized form several mosquito species (Isoe and Hagedorn, 2007). Work by Tsang et al. (2003) show that the shrimp *Metapenaeus ensis* has and expresses at least two Vgs. Four Vgs are known from the toad *Xenopus laevis* (Wahli et al., 1981). A study by Wahli et al. (1981) suggested multiple Vg genes have arise by gene duplication. Multiple Vgs may confer an advantage, but is not clear if the functions of Vgs in a particular species are the same or differ. Tufail and Takeda (1998) suggested different Vg genes may have different functions. The role of Vg as the major yolk protein is to provide nutrients to the developing embryo, but Vg may also have other roles; for example, different behavior is observed in sterile worker

honey bees in correlation with Vg levels in their hemolymph (Amdam et al., 2003). The role of Vg in immature and male stages is not known and it should be investigated. It also remains unclear if multiple Vgs interact with one VgR or if there are multiple VgRs as well.

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Table 2. Forward and reverse primers designed based on the DNA-sequence of contigs similar to vitellogenin (Vg), vitellogenin receptor (VgR) and lysozyme. The length of the amplified fragment and the annealing temperature used during PCR is included.

Contig	Primer Sequence			
Putative Vg	Forward	Reverse	Amplified fragment size	Annealing temperature (°C)
00558	5'-CAGCAGAGCGGTGCTACCTACAAGG-3'	5'-CTCGACCTTGAGGTTCTTGCCGT-3'	213	68
08149	5'-TCCGCCGTAACGCTTCTCCTGA-3'	5'-AGAACCGTCGCATCGAGGGTGT-3'	236	68
10299	5'-GAAGGAACCGGTGAGCTCGAAAG-3'	5'-TTTCCACGTCGAGCCCACCA-3'	154	68
11791	5'-TTGAGGTGCTGGTTGAGCTTCT-3'	5'-TCGTCGCTCCCAAGTACAAG-3'	210	63
11824	5'-GTGGCCGCCGGAAAGATCAT-3'	5'-CTTGGGAAAGGCGCTCAACGTA-3	214	68
12365	5'-GGAGACACTAACTTGAGCGGACG-3'	5'-GCACCGAACAAGTCGAGTCC-3'	227	66
Putative VgR				
00618	5'-CAATCGCTGCTTCAGAAATCAGTGC-3'	5'-TTCACATAGGTCTCCGTGGAAGCC-3'	256	69
08209	5'-AACGCTGCACTCACTCTGAGCATG-3'	5'-TCCGCATTTGAGGACCGCAG-3'	219	69
09459	5'-AAGACTCGTGCTTGTATTCCCCGTG-3'	5'-TCGAAAGCGGTGATCCCATCAC-3'	222	69
Lysozyme				
05664	5'-ATCGACGCCGGAAGACCTGGAAAC-3'	5'-AACAAGTGACAGCTCCGTCGCCGT-3'	140	72

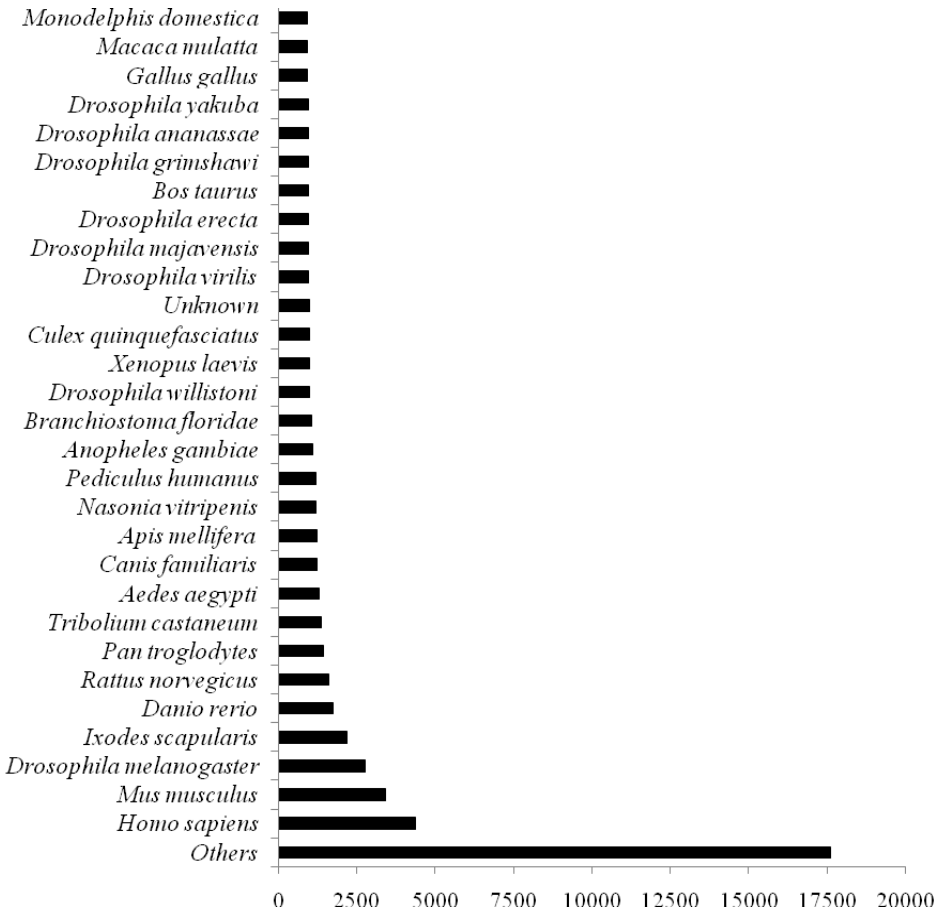

Figure 1. Species distribution of transcript sequences from a whole-body *Phytoseiulus persimilis* cDNA library with significant homology to searches from NCBI non redundant database.

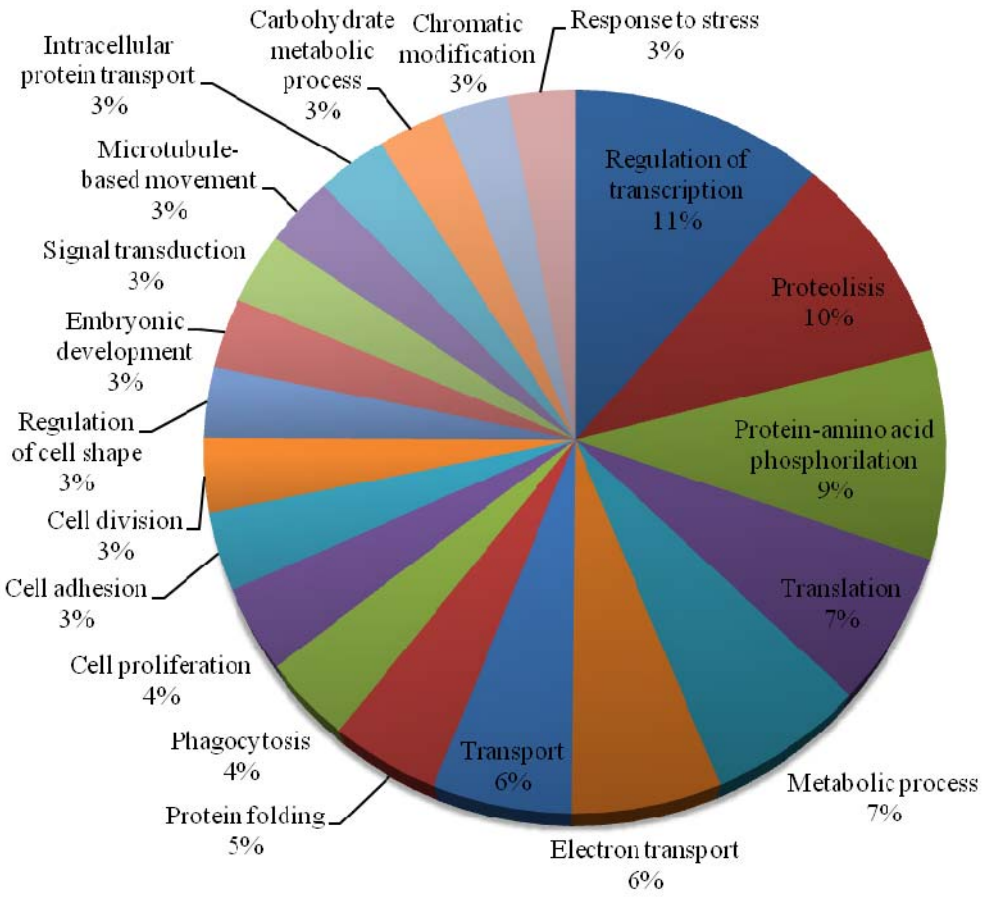

Figure 2. Distribution of transcripts from a whole-body *Phytoseiulus persimilis* transcriptome within major categories of level 2 biological functions, gene ontology (GO) analysis (n= 630 contigs).

Table 2. The 50 most abundant transcripts from a transcriptome analysis of whole-body, mobile stages of *Phytoseiulus persimilis*.

Transcript	Counts	Best Match to NR protein data	Accession no.	Species	E-value	Conserved domains	Putative function
11492	861	hypothetical protein BRAFLDRAFT_121184	XP_002601749.1	*Branchiostoma floridae*	1e-07	none	unknown
12538	794	similar to serine/threonine-protein kinase	XP_001607687.1	*Nasonia vitripennis*	1e-123	PKc_like	molecular function
11428	715	unknown				none	unknown
11503	687	unknown				none	unknown
11516	570	unknown				none	unknown
11493	565	unknown				none	unknown
11515	537	unknown				none	unknown
11434	517	unknown				none	unknown
11485	516	unknown				none	unknown
00358	511	hypothetical protein BRAFLDRAFT_126153	XP_002609128.1	*Branchiostoma floridae*	0.0	NAD_binding, Inos-1-P synth	Inositol phosphate metabolism
10386	492	unknown				none	unknown
00426	466	retinoblastoma-binding protein	NP_001072026.1	*Ciona intestinalis*	8e-18	ARID, CHROMO	Ras mediated signal transduction
11526	455	unknown				none	unknown
10639	451	hypothetical protein SIAM614_05486	ZP_01550994.1	*Stappia aggregata*	1.3	none	unknown

Table 2. Continued.

11795	450	hypothetical protein AaeL_AAEL012930	XP_001663118.1	*Aedes aegypti*	2e-66	NOT_2_3_5	RNA degradation
11500	445	40S ribosomal protein S13	ABI52720.1	*Argas monolakensis*	4e-11	Ribosomal_S13_N	ribosomal small subunit biogenesis and assembly
11522	445	unknown				none	unknown
12389	444	cell adhesion molecule, putative	XP_002410949.1	*Ixodes scapularis*	2e-05	FN3	cell adhesion molecule activity
11364	442	unknown				none	unknown
09479	441	unknown				none	unknown
11513	441	unknown				none	unknown
12342	435	phosphatidylinositol-4-phosphate 5-kinase type II, putative	XP_002405104.1	*Ixodes scapularis*	2e-34	PIPKc	Inositol phosphate metabolism, phosphatidylinositol signaling system
10436	434	arabinan endo-1,5-alpha-L-arabinosidase A	EER44850.1	*Ajellomyces capsulatus*	0.93	none	Catalysis of the endohydrolysis of 1,5-alpha-arabinofuranosidic linkages in 1,5-arabinans
08292	395	unknown				none	unknown
12317	390	conserved hypothetical protein	XP_002427653.1	*Pediculus humanus*	6e-119	none	unknown
10991	388	translation initiation factor 2C, putative	XP_002405664.1	*Ixodes scapularis*	1e-53	Piwi-like	translation factor activity, nucleic acid binding

Table 2. Continued.

11510	388	ATP-dependent RNA helicase	ZP_01900811.1	*Moritella sp.*	6.4	none	Catalysis of the reaction: ATP + H2O = ADP + phosphate, driving the unwinding of an RNA helix.
11796	387	guanine deaminase	ZP_01612659.1	*Alteromonadales bacterium*	7.7	none	guanine aminohydrolase activity
10645	383	nanos 2	AAU11514.1	*Podocoryna carnea*	3e-12	zf-nanos	RNA binding
11486	383	unknown				none	unknown
03129	376	unknown				none	unknown
11527	376	unknown				none	unknown
00578	371	ubiquitin protein ligase, putative	XP_002408338.1	*Ixodes scapularis*	1e-84	UBCc	anaphase-promoting complex activity
11791	371	Vitellogenin	AAW78557.2	*Dermacentor variabilis*	3e-28	von Willebrand factor D	lipid transport
00041	367	replicase polyprotein	NP_044945.1	*Drosophila C virus*	1e-57	RNA_dep_RNAP,. RT_like	virus replication
11507	365	unknown				none	unknown
00836	363	adenylate cyclase, putative	XP_002433963.1	*Ixodes scapularis*	8e-96	MFS	ion transportation across cytoplasmic or membranes
02217	363	signal recognition particle receptor, alpha subunit	XP_002410250.1	*Ixodes scapularis*	5e-48	P-loop NTPase	cellular component

Table 2. Continued

12303	359	PREDICTED: similar to GA16037-PA	XP_001605640.1	*Nasonia vitripennis*	0.0	COG4886, Exo_endo_phos	unknown
01329	358	similar to GA15568-PA	XP_001601463.1	*Nasonia vitripennis*	4e-108	WD40	Ubiquitin-mediated oocyte maturation
01030	357	Cniwi protein, putative	XP_002399390.1	*Ixodes scapularis*	7e-34	Piwi-like	dorso-ventral axis formation
12211	348	unknown				none	unknown
12284	348	conserved hypothetical protein	XP_002406447.1	*Ixodes scapularis*	7e-21	MADF_DNA_ bdg, BESS	unknown
12401	347	soluble cytochrome b562	ZP_05927222.1	*Vibrio sp*	0.046	none	unknown
00240	345	conserved hypothetical protein	EEQ32392.1	*Microsporum canis*	9.5	none	unknown
12145	345	GI21552	XP_002010719.1	*Drosophila mojavensis*	0.004	none	Assembly reconciliation
00654	344	similar to AGAP006083-PB [	XP_974501.2	*Tribolium castaneum*	9e-45	FN3	unknown
01700	338	unknown				none	unknown
01901	336	predicted protein	XP_001417385.1	*Ostreococcus lucimarinus*	2e-08	P-loop, NTPase	unwinding of a DNA or RNA helix
11506	336	unknown				none	unknown

Table 3. Transcripts from *Phytoseiulus persimilis* with similarity to vitellogenin amino acid sequence.

Contig	Length (nt)	ORF	Conserved motifs	% ID	Top match	E-value
558	615	2	VWD	33%	Vitellogenin, *Ornithodoros moubata*	7.00E-18
03748	732	-1	Vitellogenin_N	25%	Hemelipoglycoprotein precursor, *Dermacentor variabilis*	4.00E-18
08149	456	-3		34%	Vitellogenin 2 precursor, *D. variabilis*	6.00E-05
10299	205	-2		47%	Vitellogenin, *D. variabilis*	2.00E-09
10378	335	2		57%	Vitellogenin, *O. moubata*	3.00E-05
11475	262	-1	VWD	39%	Vitellogenin, *O. moubata*	4.00E-07
11712	434	3		47%	Vitellogenin, *Ixodes scapularis*	4.00E-09
11791	863	-1	VWD	34%	Vitellogenin, *D. variabilis*	3.00E-28
11824	398	2		52%	Vitellogenin, *I. scapularis*	9.00E-16
12281	440	1		34%	GP80 precursor, *Boophilus microplus*	7.00E-13
12365	2310	-2	VWD	26%	Vitellogenin, *D. variabilis*	2.00E-45

Table 4. Transcripts from *Phytoseiulus persimilis* with similarity to vitellogenin receptor amino acid sequence.

Transcript	Length (nt)	ORF	Conserved motifs	% ID	Top match	E-value
00618	618	2		40%	Low density lipoprotein related protein 2, *Danio rerio*	9.00E-12
03434	1356	-2	LDL_receptor_b	59%	GE15450, *Drosophila yakuba*	4.00E-146
06415	586	1	LDL_receptor_b	55%	Low density lipoprotein receptor-related protein 6, *Bos taurus*	6.00E-44
07393	665	-3	LDL receptor b	60%	Low density receptor related protein, *I. scapularis*	3.00E-75
08209	446	2	LDLa	51%	Hypothetical protein BRAFLDRAFT_104841	2.00E-12
09459	176	-3	EGF_CA	38%	Predicted similar to CG12139-PB	5.00E-12

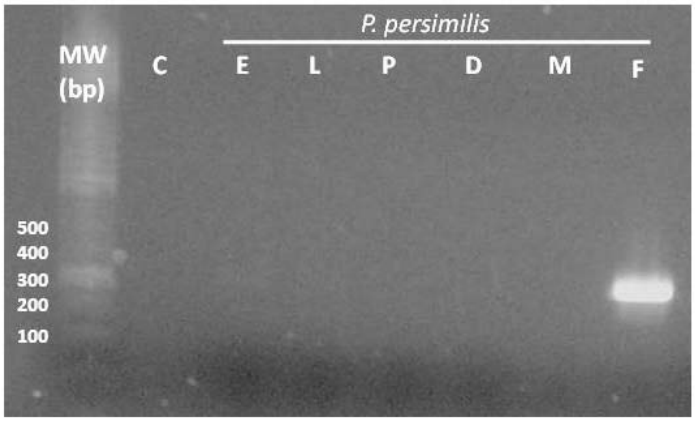

Figure 3. Expression of putative Vg (contig12365) from *Phytoseiulus persimilis*. Total RNA was extracted from single mites of different life stages, and used to synthesize cDNA that served as template for PCR. The fragment had an expected size of approximately 227 bp and was amplified only from the female adult sample. MW: Molecular weight DNA ladder, C: control, no cDNA, E: egg, L: larva, P: protonymph, D: Deutonymph, M: male adult, F: female adult.

Table 5. Stage-specific expression of selected transcripts from *Phytoseiulus persimilis* with similarity to vitellogenin (Vg) and vitellogenin receptor (VgR) (n=3).

Transcript	Life stage					
Putative Vg	Egg	Larva	Protonymph	Deutonymph	Male	Female
00558	X*	X	X	X	X	X
08149	X	X	X	X		X
10299	X	X	X			X
11791	X	X	X	X		X
11824	X					X
12365	X					X
Putative VgR						
00618	X					X
08209	X					X
09459	X					X
Lysozyme						
05664		X	X	X	X	X

* Black X indicates that the expected fragment was present in all replicates with intense brightness, dark grey X indicates the expected fragment was present in all replicates but with faded brightness, light grey X indicates expected fragment was present in one or two replicates with faded brightness.

Figure 4. Amino acid sequences towards the C-terminus containing the Von Willebrand factor D motif of translated putative vitellogenins (Vgs) from *Phytoseiulus persimilis* (contig11791, contig12365) compared to Vgs from the American dog tick *Dermacentor variabilis* (DvVg; AAW78557.2) and the deer tick *Ixodes scapularis* (IsVg; XP_002415224.1). Identical residues at the same positions are highlighted in light grey. The conserved motif GI/LCG, found in most insect and tick Vgs is highlighted in black.