Article

ECDYSTEROID AND CHITINASE FLUCTUATIONS IN THE WESTERN TARNISHED PLANT BUG (*Lygus hesperus*) PRIOR TO MOLT INDICATE ROLES IN DEVELOPMENT

Colin S. Brent
Arid Land Agricultural Center, USDA-ARS, Maricopa, Arizona, USA

Meixian Wang
Arid Land Agricultural Center, USDA-ARS, Maricopa, Arizona, USA and College of Animal Sciences, Zhejiang University, Hangzhou, People's Republic of China

Yun-Gen Miao
College of Animal Sciences, Zhejiang University, Hangzhou, People's Republic of China

J. Joe Hull
Arid Land Agricultural Center, USDA-ARS, Maricopa, Arizona, USA

*Vital physiological processes that drive the insect molt represent areas of interest for the development of alternative control strategies. The western tarnished plant bug (*Lygus hesperus *Knight) is a pest of numerous agronomic and horticultural crops but the development of novel control approaches is impeded by limited knowledge of the mechanisms regulating its molt. To address this deficiency, we examined the fundamental relationship underlying the hormonal and molecular components of ecdysis. At 27°C* L. hesperus *exhibits a temporally controlled nymph–adult molt*

Supporting Information is available in the online issue at wileyonlinelibrary.com.
CSB and MW are co-first authors.
Grant sponsor: China Scholarship Council (to MW); Grant sponsor: Cotton, Inc. (to CSB and JJH); Grant number: 12-373.
Correspondence to: Colin S. Brent, Arid Land Agricultural Center, USDA-ARS, Maricopa, AZ 85138.
E-mail: colin.brent@ars.usda

ARCHIVES OF INSECT BIOCHEMISTRY AND PHYSIOLOGY, Vol. 92, No. 2, 108–126 (2016)
Published online in Wiley Online Library (wileyonlinelibrary.com).
 DOI: 10.1002/arch.21322

that occurs about 4 days after the final nymph–nymph molt with ecdysteroid levels peaking 2 days prior to the final molt. Application of exogenous ecdysteroids when endogenous levels had decreased disrupted the nymphal–adult molt, with treated animals exhibiting an inability to escape the old exoskeleton and resulting in mortality compared to controls. Using accessible transcriptomic data, we identified 10 chitinase-like sequences (LhCht), eight of which had protein motifs consistent with chitinases. Phylogenetic analyses revealed orthologous relationships to chitinases critical to molting in other insects. RT-PCR based transcript profiling revealed that expression changes to four of the LhChts was coordinated with the molt period and ecdysteroid levels. Collectively, our results support a role for ecdysteroid regulation of the L. hesperus *molt and suggest that cuticle clearance is mediated by LhCht orthologs of chitinases that are essential to the molt process. These results provide the initial hormonal and molecular basis for future studies to investigate the specific roles of these components in molting.* © *2016 Wiley Periodicals, Inc.*

Keywords: Western tarnished plant bug; *Lygus hesperus*; molt; ecdysteroid; chitinase

INTRODUCTION

The plant bugs (Hemiptera: Miridae) are represented by numerous polyphagous agricultural pests, many of which have global distributions (Wheeler, 2001; Schuh, 2013). An economically important genus of this group is *Lygus*. Although control strategies for members of this genus have relied on broad-spectrum insecticides, there are increasing instances of field populations becoming resistant to many of these compounds (Snodgrass, 1996; Snodgrass and Scott, 2002; Snodgrass et al., 2009). A promising area for developing alternative insect control strategies is in targeting mechanisms that regulate major life processes. Hampering efforts toward devising such approaches for *Lygus* is a generally poor understanding of underlying physiological mechanisms. One developmental event with great potential for disruption is molting, a time when insects are particularly susceptible to environmental stressors, predation (Wheeler, 2001), and hypoxia (Camp et al., 2014). As is typical for other true mirids, *Lygus* bugs molt through five nymphal instars before eclosing as winged adults (Wheeler, 2001). Among these events, the molt to adult involves the most radical tissue reorganization and provides perhaps the best opportunity to study the underlying mechanisms.

For most insects, a molt is initiated when sufficient endogenous resources have been accumulated to trigger the production of a new cuticle. There is variation in the specifics of this process, but a general pattern has emerged based on research with key species such as *Bombyx mori, Drosophila melanogaster*, and *Manduca sexta* (Riddiford et al., 2003; Zitnan and Adams 2005). Controlling the molt is the brain, which integrates numerous somatic signals to decide when a nymphal stadium is at an end. The brain secretes prothoracicotropic hormone, which triggers the production and release of ecdysteroids from the prothoracic glands. A rapid rise and subsequent decline in ecdysteroid levels initiates the process of apolysis, the detachment of the cuticle from the epidermis. Elevated ecdysteroids induce gene expression changes in the epidermal cells, leading to the secretion of molting fluid enzymes that dissolve the inner layers of the old cuticle. The

chitin and proteinaceous components of the cuticle are desclerotized, so that they can be resorbed and utilized to construct the new cuticle (Merzendorfer and Zimoch, 2003). As the old cuticle is degraded, an apolysial space forms beneath the cuticle and fills with molting fluid. Epidermal cells then proliferate and the new cuticle is produced. When the new cuticle is in place, molting fluid is resorbed and the insect initiates a behavioral sequence that leads to ecdysis of the old cuticle. The high ecdysteroid levels that initiate the molt also help to prevent premature ecdysis (Truman, 2005). Eclosion is followed by sclerotization and pigmentation of the new cuticle.

A key part of apolysis is the secretion of chitinases by the epidermis to digest the structural polysaccharide that gives the insect exoskeleton much of its toughness and pliability (Kramer and Koga, 1986; Kramer and Muthukrishnan, 2005). Chitinases are classified as glycosol hydrolases and belong to multigene families (Henrissat and Bairoach, 1993). During the molt, chitinases are found in the molting fluid and are responsible for hydrolyzing chitin in the old cuticle into oligosaccharides (Doucet and Retnakaran, 2012). The reduced product is then further degraded by other enzymes (Fukamizo and Kramer, 1985; Filho et al., 2002), particularly β-*N*-acetylglucosaminidases, which convert to monomers the chitooligomers produced by chitinases (Merzendorfer and Zimoch, 2003). The expression levels of chitinases typically respond to circulating ecdysteroids; the increase in ecdysteroids prior to the molt results in an increase in chitinases, and the subsequent decline in the hormone reduces the enzyme levels (Kramer and Muthukrishnan, 2005). For some species it has been shown that reduction of circulating ecdysteroids through use of ligatures (Fukamizo and Kramer, 1987) or injected agonists (Zheng et al., 2003) can prevent chitinase synthesis and thereby delaying the molt. However, ecdysteroids are not the only known regulator of chitinase expression. In the darkling beetle *Tenebrio molitor*, juvenile hormone (JH) level has been shown to be the primary determinant of chitinase expression (Royer et al., 2002). The genomes of several species code for multiple chitinases (Zhu et al., 2008a; Nakabachi et al., 2010; Zhang et al., 2011; Pan et al. 2012; Xi et al., 2015), and while many are likely to have overlapping roles, their specific function can vary, as well as the timing and location of their expression (Zhu et al., 2008b; Xi et al., 2015). In addition to their role in molting, chitinases are involved in digestion, immune response, and pathogenicity (Zhu et al., 2008b; Arakane and Muthukrishnan, 2010). The varied capabilities of the chitinases are a result of the diversity in their form. While all are multidomain structures, with at least one domain allocated to catalysis and another to substrate binding (Doucet and Retnakaran, 2012), they operate with different efficiencies and target different substrates. Knockdown of these chitinases has been shown to interfere with all or parts of the molting cycle (Zhu et al., 2008b; Zhang et al., 2012; Xi et al., 2015; Li et al., 2015), making these enzymes promising targets for novel control measures. In support of such an approach, transgenic and recombinant expression of chitinases in plants has been shown to increase resistance to several pests (Zhu et al., 1994; Regev et al., 1996; Kramer et al., 1996; Ding et al., 1998; Lawrence and Novak, 2006).

Little is known about the hormonal and molecular control of molting in the western tarnished plant bug (*L. hesperus* Knight). *Lygus hesperus* is the dominant pest *Lygus* species in the western United States (Schwartz and Footit, 1998) and is damaging to numerous crops (Scott, 1977). Although the pacing of *L. hesperus* development can be influenced by exogenous factors such as diet (Debolt, 1982), population density (Brent, 2010), and temperature (Champlain and Butler, 1967; Butler and Wardecker, 1971; Cooper and Spurgeon, 2012, 2013), nothing is known of the coordination of these responses. This study provides the first detailed information on the underlying mechanisms regulating the adult molt of *Lygus*. We identified genes of the chitinase-like family in *L. hesperus*,

annotated transcriptome sequences, and mapped phylogenetic relationships. We also tracked changes before and after the adult molt to the expression of the chitinase-like sequences and circulating levels of ecdysteroids, and explored linkages to the molting process through hormonal manipulation. These are important initial steps toward developing control approaches that target a potential vulnerability of this key pest.

MATERIALS AND METHODS

Insect Rearing

Lygus hesperus were obtained from an in-house colony (USDA-ARS Arid Land Agricultural Research Center, Maricopa, AZ). Stock insects were given unrestricted access to a supply of green bean (*Phaseolus vulgaris* L.) pods and an artificial diet mix (Debolt, 1982) packaged in Parafilm M (Pechiney Plastic Packaging, Chicago, IL; Patana, 1982). Both food sources were replenished as needed. Insects were reared at 27.0 ± 1.0°C, 40–60% relative humidity, under a 14:10 L:D photoperiod. Experimental insects were generated from eggs deposited in oviposition packets (agarose covered in Parafilm M) and maintained under the same conditions.

Determination of Developmental Timing and Ecdysteroid Titer

Nymphs were collected from the stock colony as fourth instars. Collected nymphs were reared in 1890-ml waxed chipboard cups (Huhtamaki, De Soto, KS) at a density known to have minimal effect on *L. hesperus* development (100–150 nymphs/container; Brent, 2010). Both genders were maintained together on a green bean and raw sunflower seed (*Helianthus annuus* L.) diet, which was replaced every 2 days. Rearing cups were covered with nylon mesh to ensure air circulation and light exposure. Daily monitoring allowed fifth-instar nymphs to be collected within 24 h of eclosion. To determine the duration of the fifth nymphal stadium and the timing of the adult molt, a group of 100 newly eclosed animals were surveyed at each 24-h interval to observe molting and track mortality. Monitoring was discontinued after 5 days, at which point more than 90% had molted to adulthood.

To determine the whole body concentration of ecdysteroids during this period, *Lygus* were sampled on the day of eclosion to fifth instar and at 24-h intervals thereafter until 48 h after the adult molt. At each time point, 15 samples were collected for each gender. Animals were collected directly into 250 μl of cold 90% methanol (Sigma-Aldrich, St. Louis, MO) to minimize the effect of handling stress on their endocrine state. Each sample consisted of 10 pooled insects, which were thoroughly homogenized with a tissue grinder then centrifuged at 4ºC and 5,000 × *g* for 10 min. The ecdysteroid-containing methanol phase was collected and placed into a labeled 1.5-ml microcentrifuge tube. Residual ecdysteroids were collected from the pellet by repeating the extraction twice more to generate a final methanol volume of ~750 μl. Extracts were dried, re-suspended in 200 μl 90% methanol and stored at –80ºC until assayed. Ecdysteroid content was determined by a competition radioimmunoassay originally developed by Warren et al. (1984) as modified by Brent et al. (2006). Duplicate 10-μl aliquots of the methanol phase of each partitioned sample were incubated overnight with 100 μl of (3H)-20-hydroxyecdysone stock (85.2 μCi/mmol, Perkin-Elmer, Waltham, MA) in borate buffer, and 100 μl of a polyclonal ecdysteroid antiserum (H-22 antibody, L. Gilbert, UNC-CH) at

4°C on an orbital shaker. The specific ecdysteroid is unknown for *L. hesperus*, but the H-22 antibody cross-reacts with ecdysone, ecdysterone, 20-hydroxyecdysone, and makisterone A (Warren and Gilbert, 1986). Intra- and interassay variability was minimized by generating standard competition curves for each set of samples, using a 20-hydroxyecdysone (20E) stock (Sigma-Aldrich, St Louis, MO) over a range of 15.6–2000 pg. After incubating ~18 h, 20 μl of cleaned protein A solution (Pansorbin; CalBiochem, San Diego, CA) was added to each tube to precipitate the antibody–antigen complex. Tubes were incubated for 1 h at 25°C, and then centrifuged at 5,000 × *g* for 5 min. Supernatant was removed and the remaining pellet was washed twice with 100 μl of borate buffer. The incorporation of microlabel was determined by a 2450 MicroBeta2 scintillation counter (Perkin-Elmer, Waltham, MA) and ecdysteroid concentrations were estimated by nonlinear regression (Brent et al., 2006).

Manipulation of Molt Timing with Ecdysteroid Application

Lygus raised at 27ºC have a short (≤24 h) interval between the drop from peak ecdysteroid production and the adult molt. To discern differences in molt timing that are induced by exogenous hormone treatments, it was necessary to slow the rate of development to elongate this postpeak period. Such developmental delays can be achieved by rearing *Lygus* at low temperatures (Champlain and Butler, 1967; Butler, 1970; Butler and Wardecker, 1971; Cooper and Spurgeon, 2013). To determine the timing of the adult molt under a low-temperature regime, newly eclosed fifth instars were reared in an environmental chamber (model I30BLL, Percival Scientific, Perry, IA) set to 16.9 ± 1.0ºC (40–60% RH; 14:10 L:D). Once the timing of the adult molt was determined, another cohort of newly eclosed fifth instars was collected and held as described above at 16.9ºC. Six days later, when ecdysteroid levels were expected to drop to a premolt nadir, groups of 200 nymphs each received one of three treatments to test the effect of disrupting the normal decline in ecdysteroid levels. One group received a 1-μl aliquot of 20E dissolved in 90% methanol (1 μg/μl) topically applied with a micropipettor (Eppendorf Research 0.1–2.5 μl; Hamburg, Germany) to the dorsal side of abdomen. A second group received a 1-μl aliquot of 90% methanol alone. The third group was handled but untreated. Molting and mortality were recorded every 24 h after treatment until 12 days after the fifth-instar molt.

Identification and Bioinformatic Characterization of **L. hesperus** *Chitinases*

To identify putative *L. hesperus* chitinase (LhCht) sequences, we searched our previously assembled *L. hesperus* transcriptomes (Hull et al., 2013, 2014) with BLASTx (*e*-value ≤ 10^{-5}) using queries consisting of chitinase-like sequences (Supplementary Table 1) from species representing six orders: Diptera (*D. melanogaster*), Lepidoptera (*B. mori*), Hymenoptera (*Nasonia vitripennis*), Coleoptera (*Tribolium castaneum*), Phthiraptera (*Pediculus humanus corporis*), and Hemiptera (*Nilaparvata lugens*). The longest isoforms from the resulting *L. hesperus* sequence hits were then re-evaluated by BLASTx (*e*-value ≤ 10^{-5}) using the NCBI nr database with the top five BLAST hits exported in table format for annotation (Supplementary Table 2). Identification of putative chitinase domains was performed using the HMMER webserver (Finn et al., 2011) and protein databases consisting of Pfam, Gene3D, and Superfamily with cutoffs based on the gathering threshold. Prediction of transmembrane domains was performed using TMPred (Hofmann and Stoffel, 1993) and TopPred (Heijne, 1992; Claros and Heijne, 1994). Signal peptide prediction was performed with SignalP 4.0 (Petersen et al., 2011).

To determine phylogenetic relationships, MUSCLE-based multiple sequence alignments (Edgar, 2004) consisting of the putative *L. hesperus* sequences and chitinase sequences from the six species used in the initial BLASTx search were performed using default settings in Geneious 7.1.7 (Kearse et al., 2012; Biomatters Ltd., Auckland, New Zealand). Phylogenetic trees were constructed using the maximum likelihood, minimum evolution, UPGMA, and neighbor joining methods in MEGA6 v6.06 r6140220 (Tamura et al., 2013). For the maximum likelihood analysis, all positions with less than 95% site coverage were eliminated leaving a total of 198 positions in the final dataset. In the other analyses, all ambiguous positions were removed for each sequence pair leaving a total of 6,900 positions in the final datasets. The clustering of sequences and orthologous genes was compared among the phylogenetic methods and with previous phylogenetic analyses of chitinases and chitinase-like sequences (Arakane and Muthukrishnan, 2010; Nakabachi et al., 2010; Pan et al., 2012; Xi et al., 2015). A percent identity heat map for the putative *L. hesperus* chitinase sequences was generated using MUSCLE-based sequence alignment as described above.

RT-PCR Expression Profile of Chitinase-like Transcripts in L. hesperus

Expression of the *L. hesperus* chitinase-like transcripts was initially examined in pooled samples. For first and second instars, 20 nymphs of mixed gender were used. For third–fifth, 10 nymphs were pooled. For adults, five of each gender were pooled at ages of 0, 1, 3, 7, and 10 days post eclosion. Subsequent analyses utilized fifth instars at 0, 1, 2, 3, and 4 days post ecdysis, and adults of each gender sampled at 0, 1, and 2 days post eclosion. Insects were drawn from the same cohort used to determine the ecdysteroid titer. To obtain total RNAs, samples were homogenized in TRI Reagent Solution (Life Technologies, Carlsbad, CA) based on recommendations from the manufacturer using a TissueLyser (Qiagen, Valencia, CA). Isolated total RNA was quantified based on absorbance at 260 nm using a Take3 multivolume plate on a Synergy H4 hybrid multimode microplate reader (BioTek Instruments, Winooski, VT). First strand cDNA was synthesized from 500 ng of DNase I-treated total RNAs using a SuperScript III (Life Technologies) first-strand cDNA synthesis kit with custom made random pentadecamers (IDT, San Diego, CA). End-point PCR amplification was performed using Sapphire Amp Fast PCR Master Mix (Takara Bio Inc./Clontech, Palo Alto, CA) with 0.4 μl (10 ng) cDNA template and sequence-specific primers (Supplementary Table 3) designed to amplify ~500 bp fragments of the *L. hesperus* chitinase-like transcripts or a 555-bp fragment of the *Lygus* actin transcript (accession no. DQ386914). Thermocycler conditions consisted of 95°C (2 min), followed by 35 cycles of amplification (94°C, 20 sec; 56°C, 20 sec; 72°C, 20 sec) and a final extension of 72°C for 5 min. Expression profiles were replicated at least three times using cDNA templates prepared from different biological replicates. PCR products were electrophoresed on 1.5% agarose gels and visualized with SYBR Safe (Life Technologies). Representative bands were subcloned into a pCR2.1 TOPO TA cloning vector (Life Technologies) and sequenced at the Arizona State University DNA Core Lab (Tempe, AZ).

Statistical Analyses

Female and male ecdysteroid concentrations were compared for each time point sampled using the Mann–Whitney rank-sum test, with a Bonferroni correction for multiple comparisons and $\alpha = 0.05$. Comparisons of the adult molt probabilities, and survivorship after different treatments were made by survival analyses using the log-rank statistic.

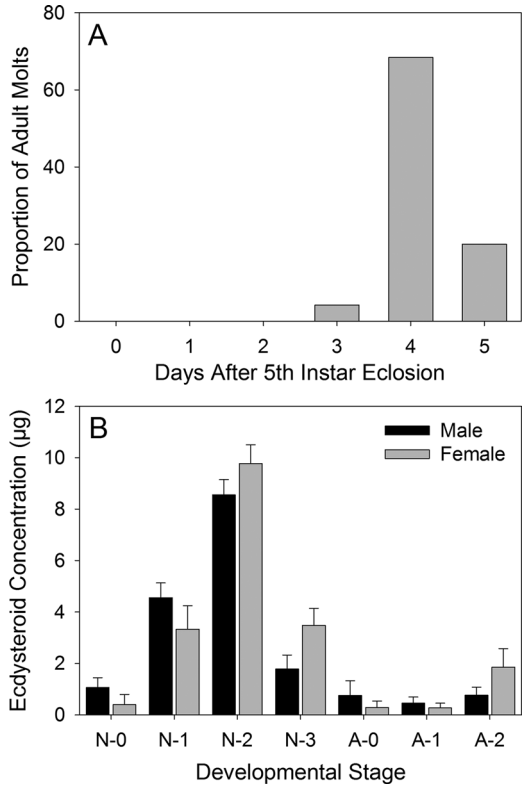

Figure 1. (A) The percent of mixed gender nymphs (N; $n = 95$) that molted into adults (A) each day subsequent to their molt to fifth instar. (B) The mean (±SE) concentration of ecdysteroids, in 20-hydroxyecdysone equivalents, for individual whole bodies during the fifth instar and early adult development of females and males. Values for females and males do no differ significantly ($P > 0.05$, Mann–Whitney rank-sum tests; Bonferroni's correction for multiple comparisons). There are 15 samples per sex, per sample period.

Animals that died prior to molting or failed to molt prior to the end of the sample period were included in the molting analysis as censored data. Likewise, animals still alive by the end of the sample period were included in the mortality analysis as censored data. The Holm–Sidak method was used for pairwise multiple comparisons of survival curves, using $\alpha = 0.05$. Analyses were conducted with Sigmaplot 11.0 (Systat Software, Point Richmond, CA, USA).

RESULTS

Developmental Timing and Ecdysteroid Titer

The first molts to adulthood recorded for *Lygus* reared at 27°C occurred by 3 days after the molt to fifth instar, but the average time to molt was 4.2 ± 0.5 days post ecdysis (Fig. 1A). The molt was preceded by an increase in the whole body concentration of ecdysteroid for both females and males (Fig. 1B). Compared to hormone concentrations observed just after the molt to the fifth instar, ecdysteroids increased by day 1, peaked with an eightfold to 12-fold increase on day 2, and declined to a basal level by day 3.

In contrast to the nymphs, ecdysteroid titers were generally low in the adults, although the concentration was somewhat higher for 3-day-old females. There were no significant differences in concentration between females and males for any of the sample times (Mann–Whitney, $P > 0.05$).

Response to Ecdysteroid Application

Nymphs reared at 16.9°C developed more slowly than those reared at 27°C, with a mean stadium length of 8.6 ± 1.0 days for the fifth instar. From this doubling of development time, it was estimated that the highest concentration of ecdysteroids would occur ~4 days post eclosion, and that the subsequent premolt decline would be initiated by day 5. Therefore hormonal manipulation was applied 5 days after molt to the fifth instar. Treatment had a pronounced effect on molt timing (Fig. 2A; $\chi^2_{logrank} = 100.5$, df = 2, $P < 0.001$). Topical application of 20E resulted in a model-estimated mean stadium length of 10.0 ± 0.1 days, which was significantly longer than the interval for nymphs treated with MeOH (9.0 ± 0.1 days; Holm–Sidak; $\chi^2_{logrank} = 75.40$, df = 1, unadjusted-$P < 0.001$), or left untreated (9.0 ± 0.1 days; $\chi^2_{logrank} = 69.82$, df = 1, unadjusted-$P < 0.001$). There was no significant difference between the latter two groups ($\chi^2_{logrank} = 0.59$, df = 1, unadjusted-$P = 0.44$). In addition to lengthening nymphal development time, 20E application also influenced mortality (Fig. 2B; $\chi^2_{logrank} = 123.24$, df = 2, $P < 0.001$). Individuals treated with the 20E had lower survival rates compared to those given MeOH (Holm–Sidak; $\chi^2_{logrank} = 90.62$, df = 1, unadjusted $P < 0.001$) or no treatment (Holm–Sidak; $\chi^2_{logrank} = 64.48$, df = 1, unadjusted-$P < 0.001$). Survival did not differ between the latter two groups ($\chi^2_{logrank} = 3.78$, df = 1, unadjusted-$P = 0.052$). While deaths were evenly distributed throughout the sample period for the latter two groups, 91.5% of the 20E-associated mortality occurred near the time of the adult molt. Most insects that died during the molt were trapped in the exuvium.

Identification of Chitinase-like Transcripts in L. hesperus

To identify putative chitinase-like sequences in *L. hesperus*, we performed a tBLASTn search of previously assembled transcriptomes (Hull et al., 2013, 2014) using chitinase-like sequences from six insect species (*D. melanogaster*, *T. castaneum*, *N. vitripennis*, *P. humanus*, *B. mori*, and *N. lugens*) as queries. The longest transcripts identified from that search were then used as queries in BLAST searches of the NCBI nr database (Supplementary Table 2). Taken together, the BLAST analyses suggest the presence of 10 chitinase-like sequences (GenBank accession nos. KT717326-KT717335) in *L. hesperus*; eight chitinases (LhCht1-8) and two chitinase-like sequences encoding a putative endo-beta-*N*-acetylglucosaminidase (LhENGase) and an imaginal disc growth factor (LhIDGF1), respectively. Based on in-frame start and stop codons, eight of the transcripts encompass complete open reading frames (ORFs) whereas the other two transcripts correspond to a 5′ fragment containing a putative start codon (LhCht2) and a 3′ fragment containing a putative stop codon (LhCht8). The presence of glycosyl hydrolase family 18 or chitin-binding peritrophin-A signature motifs in nine of the transcripts (Supplementary Table 4) as well as signal peptides (Supplementary Table 4) in seven of the sequences supports the initial chitinase annotations. While the putative LhENGase lacks these features, it has motifs and domains consistent with endo-beta-*N*-acetylglucosaminidases (e.g., glycosyl hydrolase family 85; lack of signal peptide) that hydrolyse the *N*,*N*′-diacetylchitobiosyl core of *N*-glycosylproteins and are part of the GH18 chitinase-like superfamily (Lombard et al., 2014). The absence

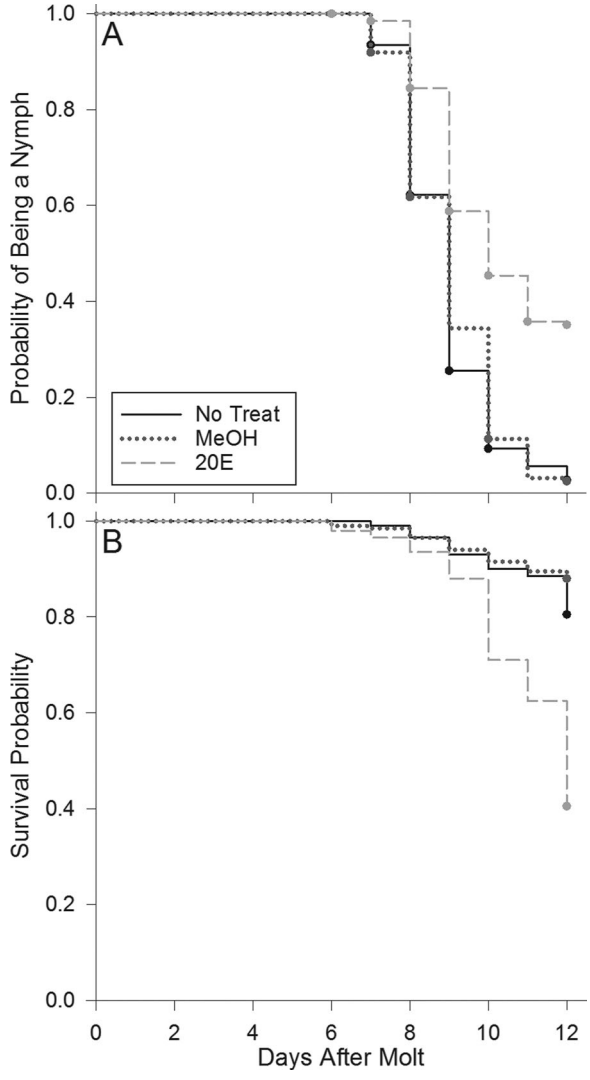

Figure 2. The proportion of fifth-instar nymphs that have not yet gone through the adult molt after topical application of 20-hydroxyecdysone (20E) or the 90% methanol carrier (MeOH), or after receiving no treatment (No treat; $n = 200$ for each). Those treated with 20E molted later and in lower overall proportion compared to nymphs of the other groups ($P < 0.05$, Gehan–Breslow statistics for the survival curves; post hoc comparisons with the Holm–Sidak method for all pairwise multiple comparisons). Circles represent individuals that died prior to the adult molt or that were still alive but had not molted by the end of the sample period.

of a signal peptide in LhCht8 is not unexpected as it is a 3′ fragment that lacks a start codon. Previous studies (Zhu et al., 2004; Arakane and Muthukrishnan, 2010; Nakabachi et al., 2010) have reported four conserved regions (CR) in insect chitinases: CR1—KXXXXXGGW (X signifies any amino acid); CR2—FDGXDLDWEYP; CR3—MXYDXXG, and CR4—GXXXWXXDXDD. CR2 corresponds to the catalytic region of the enzyme with the conserved glutamic acid (E) in this motif thought to be essential for chitinase activity. The four regions are moderately conserved in the *L. hesperus* chitinase-like sequences (Supplementary Table 5). However, the critical glutamic acid in the CR2 motif of LhCht3

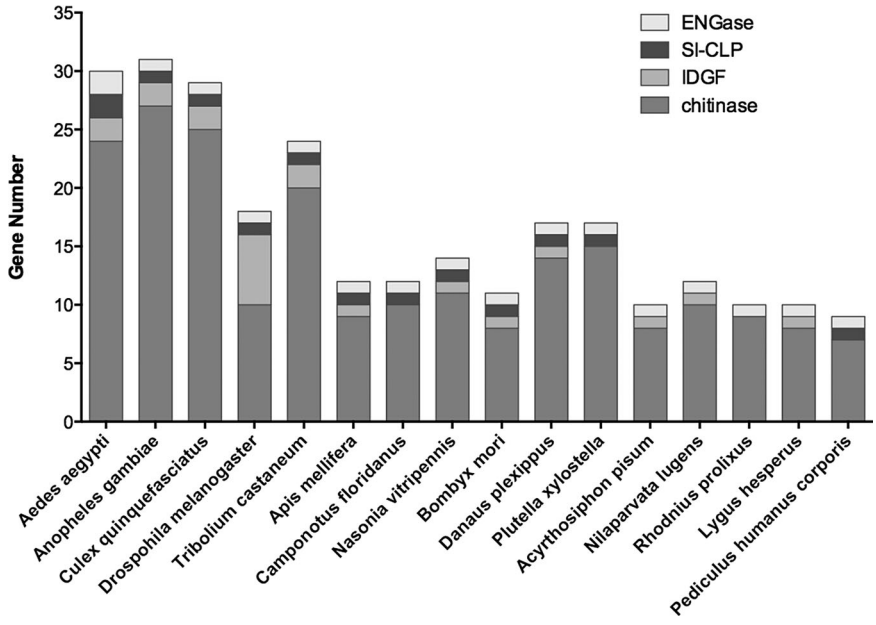

Figure 3. Distribution of chitinase-like genes in multiple insect species. Genes and/or transcripts are derived from 16 species of insects representing six orders (Diptera, Coleoptera, Hymenoptera, Lepidoptera, Hemiptera, and Phthiraptera). ENGase, endo-beta-*N*-acetylglucosaminidase; SI-CLP, stabilin-1 interacting chitinase-like protein; IDGF, imaginal disc growth factor. Values are based on numbers reported in Nakabachi et al. (2010), Pan et al. (2012), and Xi et al. (2015).

has been replaced with a glutamine residue suggesting impaired chitinase activity. Similar to other chitinase-like proteins, sequence identity among the *L. hesperus* sequences is extremely limited, ranging from 4 to 28% (Supplementary Table 6). The 10 *L. hesperus* chitinase-like sequences identified may underrepresent the full complement of chitinases due to exclusion of temporally or spatially restricted transcripts not captured in the transcriptome sequencing. The number, however, is comparable to that reported for other hemimetabolous species (Nakabachi et al., 2010; Xi et al., 2015; Fig. 3).

Phylogenetic Analyses of L. hesperus *Chitinase-like Transcripts*

To further characterize the 10 chitinase-like proteins in *L. hesperus*, molecular phylogenetic analyses were performed using the complete predicted protein sequences and chitinase-like sequences from the six insect species used in the initial tBLASTn searches (see Supplementary Table 1). Phylogenetic trees were constructed using maximum likelihood, neighbor-joining, UPGMA, and minimum evolution methods with all exhibiting similar topologies (data not shown). Similar to previous reports (Nakabachi et al., 2010; Xi et al., 2015), the chitinase-like sequences clustered into 10 central clades (Fig. 4), labeled according to Nakabachi et al. (2010). Among the *L. hesperus* sequences, none clustered within the stabilin-1 interacting chitinase-like protein (SI-CLP) clade, which consists of glycosyl hydrolase 18 superfamily proteins that lack chitinase activity. The absence of SI-CLP sequences in *L. hesperus* is consistent with findings for other hemipteran insects, including *Acyrthosiphon pisum*, *Rhodnius prolixus*, and *N. lugens* (Nakabachi et al., 2010; Xi et al., 2015). The presence of a single SI-CLP-like sequence in *P. humanus* (EEB15598),

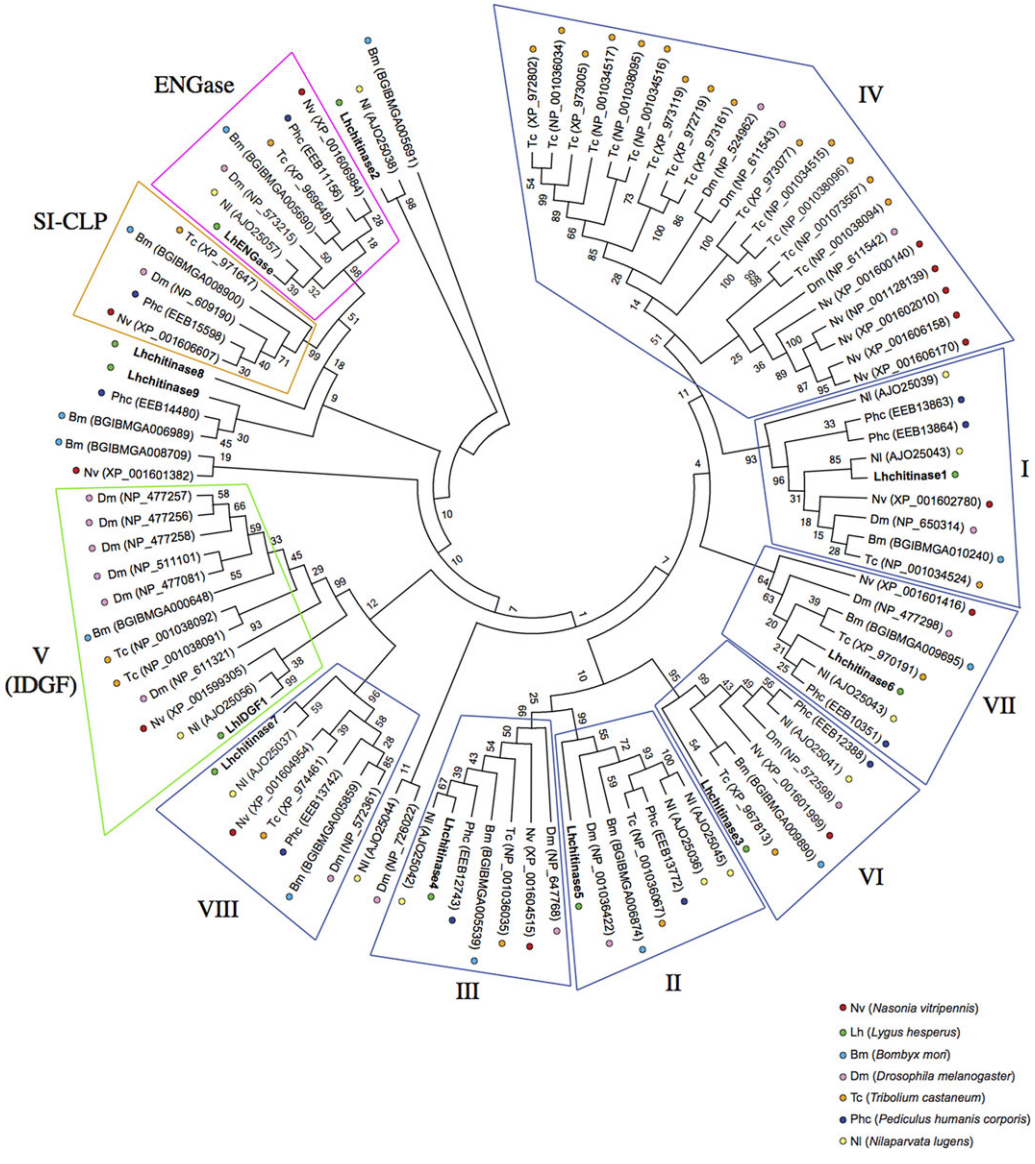

Figure 4. Phylogenetic relationships of chitinase-like proteins from *L. hesperus*. Putative *L. hesperus* chitinase-like protein sequences and full length chitinase-like sequences from six insect species were aligned using MUSCLE (Edgar, 2004). The evolutionary history was inferred using the maximum likelihood method implemented in MEGA6.0 (Tamura et al., 2013). The bootstrap consensus tree inferred from 1,000 replicates represents the evolutionary history of the taxa analyzed. Numbers at branch points indicate bootstrap support values. ENGase, endo-beta-*N*-acetylglucosaminidase; SI-CLP, stabilin-1 interacting chitinase-like protein; IDGF, imaginal disc growth factor. Boxes indicate particular clades: green, IDGF; orange, SI-CLPA; pink, ENGase; blue, chitinase groupings based on Nakabachi et al. (2010); gray chitinase gene duplications in holometabolous insects. A full listing of the used accession numbers (indicated in parentheses) can be found in Supplementary Table 1. *Lygus hesperus* proteins are shown in bold font.

another hemimetabolous insect, suggests that loss of the SI-CLP gene may be order-specific. The eight complete *L. hesperus* ORFs each aligned to one of the major clades (Fig. 4). In contrast, the two partial sequences (LhCht2 and LhCht8) showed less sequence conservation; LhCht2 grouped with two other sequences on a minor branch adjacent to the IDGF clade and LhCht8 segregated independently. This divergence was not specific to LhCht8, as four other sequences also failed to cluster within a defined group. Chitinase-like sequences that fail to resolve phylogenetically have been reported previously (Nakabachi et al., 2010; Xi et al., 2015), suggesting they may be unique to the species under study.

Seven of the 10 LhChts were identified as potential orthologs of sequences in *N. lugens* and *P. humanus*, both of which are hemimetabolous species. Orthologs of *N. lugens* sequences include LhCht1:NlCht5 (AJO25040), LhCht2:NlCht3 (AJO25038), LhCht4:NlCht7 (AJO25042), LhCht7: NlCht2 (AJO25037), and LhIDGF1:NlIDGF1 (AJO25056). RNAi-based knockdown of NlCht5 and NlCht7 had severe effects on molting (Xi et al., 2015), suggesting functional roles in cuticle degradation. No observable effects on morphology or survival were noted following knockdown of the other NlCht orthologs. Orthologs of *P. humanus* sequences consist of LhCht6:EEB10351 and LhENGase:EEB11156. LhCht6 and EEB10351 aligned to clade VI, the functionality of which is unknown as RNAi-mediated knockdown of NlCht8 (AJO25043) had no observable phenotype (Xi et al., 2015). No orthologs were identified for LhCht3, 5, or 8. LhCht3 clustered in clade V with sequences including a *Drosophila* chitinase (NP_572598) that functions in fly hair morphogenesis (Adler et al., 2013). LhCht5 sorts with clade II chitinases that are thought to function in separating individual chitin chains. In support of this role, knockdown of NlCht1 (AJO25036) and NlCht10 (AJO25045) resulted in lethal phenotypes with impaired development at the nymph–nymph molt (Xi et al., 2015), whereas TcCht10 (NP_001036067) is critical for completion of the larval–larval, larval–pupal, and pupal–adult molts in holometabolous species (Zhu et al., 2008b). Consistent with the phylogenetic analyses of Nakabachi et al. (2010), no chitinase sequences from any of the hemimetabolous species examined (i.e. *L. hesperus*, *N. lugens*, and *P. humanus*) sorted to clade VII, which is specific to holometabolous chitinase-like genes that have undergone significant expansion.

Expression Profile of **L. hesperus** *Chitinase-like Transcripts*

To gain insights into the potential functional roles of the putative chitinases in *L. hesperus*, we used RT-PCR to examine transcript abundance of the 10 sequences across nymphal development (i.e., first–fifth instars) and at different points in the adult life (Fig. 5A). All of the transcripts were amplified from each of the instars/ages examined but the relative intensity of the amplimers varied. Amplification of five of the sequences (LhCht1, LhCht4, LhCht7, LhCht8, and to a lesser extent LhCht3) decreased with adult age, suggesting a reduction in overall transcript abundance. In contrast, amplification of the other *L. hesperus* chitinase-like transcripts were relatively stable across the instars/ages examined. To develop a more precise understanding of transcript abundance in relation to the nymphal–adult molt, we next examined expression of the sequences at 24-h intervals from the day of eclosion to the fifth instar through 2 days post adult eclosion (Fig. 5B). Amplification of six of the 10 sequences was largely consistent across this time period. In contrast, the levels of LhCht1, LhCht2, LhCht3, and LhCht4 varied with nymphal age. LhCht1 exhibited rhythmic expression with levels decreasing one day after the fourth–fifth instar molt before increasing 24 h later (i.e., at 2 days after eclosion to the fifth

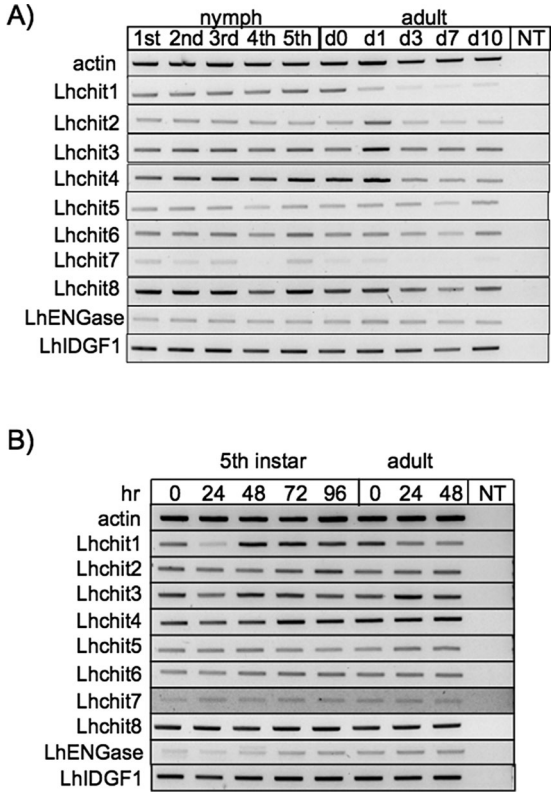

Figure 5. RT-PCR based expression profile of chitinase-like transcripts in *L. hesperus*. (A) Developmental expression profile. First- (1st), second- (2nd), third- (3rd), fourth- (4th) and fifth-instar (5th) nymphs; adults on the day of eclosion (d0); day 1- (d1), day 3- (d3), day 7- (d7), and day 10-adults (d10); and no template (NT). (B) Expression profile in fifth-instar and early adults at 24-h intervals. Amplimers represent ~500-bp fragments of the transcripts of interest. Imaging done using 1.5% agarose gels stained with SYBR Safe. For better clarity of low-expression transcripts, negative images of the gel images are shown.

instar) and remained relatively stable before decreasing again soon after adult eclosion; a profile that is reminiscent of 20E titers over the same period (Fig. 1B). The other three transcripts (LhCht2, LhCht3, and LhCht4) had profiles similar to that of LhCht1, albeit not as pronounced.

DISCUSSION

Despite the economic importance of *Lygus* bugs, little is known about the mechanisms driving molting in these species. To address this deficiency, we explored fundamental questions regarding the underlying hormonal and molecular mechanisms. In most insects, the molt is preceded by a significant increase in circulating ecdysteroid titers (Merzendorfer and Zimoch, 2003; Zitnan and Adams, 2005). Consistent with this paradigm, *L. hesperus* ecdysteroid levels peaked about 2 days prior to adult eclosion (Fig. 1B). The critical role of 20E in the *L. hesperus* molt was demonstrated with the addition of exogenous 20E one day after the occurrence of peak titers (when 20E titers normally have declined), a treatment that extended the fifth stadium and significantly increased mortality at the time of

the adult molt, with most nymphs incapable of escaping the exuvium (Fig. 2A and B). Similarly impaired molts/incomplete ecdyses in response to exogenous 20E application have been reported for other species (Kubo et al., 1981; Tanaka and Naya, 1995; Wang et al., 2012; Sun et al., 2015). The timing and duration of the ecdysteroid pulse is thus critical to the initiation of the molt. While a detailed understanding of the role of ecdysteroids exists in holometabolous insects, their function in hemimetabolous insects is not as well known. However, it is clear that 20E levels affect an intricate transcriptional regulation cascade involving multiple nuclear receptors, disruption of which can lead to poorly timed molts and impaired ecdysis (Mané-Padrós et al., 2010, 2012; Cruz et al., 2008).

A critical component of insect growth and development is molt-dependent degradation of the chitinous structures that are extensively distributed throughout ectodermal epithelial tissues, a process catalyzed by chitinases. Elevated chitinase expression has been shown to coincide with molting in *M. sexta* (Kramer et al., 1993), *B. mori* (Kim et al., 1998), *Hyphantria cunea* (Kim et al., 1998), *Spodoptera litura* (Shinoda et al., 2001), *S. exigua* (Zhang et al., 2012), *H. armigera* (Ahmad et al., 2003), *Locusta migratoria* (Li et al., 2015), and *N. lugens* (Xi et al., 2015). In this latter species, eight of the 12 putative chitinases exhibited rhythmic expression timed to both nymphal–nymphal and nymphal–adult molts. We observed a similar pattern of expression with four of the putative LhChts that coincided with molting (Fig. 5B) and which was reminiscent of changes to the ecdysteroid titer (Fig. 1B). Consistent with this observation and the transcriptional activator role of ecdysteroids in regulating the insect molt, a number of chitinases are induced by either exogenous 20E or one of its bioactive analogs (Koga et al., 1991; Kramer et al., 1993; Royer et al., 2002; Zheng et al., 2002; Yang et al., 2013; Li et al., 2015). However, the direct role of 20E in the transcriptional response may be chitinase specific. A recent study by Li et al. (2015) identified two group I chitinases (LmCht5-1 and LmCht5-2) in *Locusta migratoria*; LmCht5-2 was strictly a 20E primary-responsive gene whereas LhCht5-1 exhibited both primary and secondary 20E responses. The 20E transcriptional response may be further regulated by the microRNA (miRNA) machinery as evidenced by the role reported for miRNA-24 in regulating the *H. armigera* chitinase *hachi* (Agrawal et al., 2013). There are also likely interaction effects with circulating concentrations of JH, which has been shown in other species to downregulate (Fukamizo and Kramer, 1987; Koga et al., 1991; Kramer et al., 1993) or promote (Royer et al., 2002) chitinase gene expression.

Initially identified as solitary genes (Kramer et al., 1993; Kim et al., 1998), high-throughput sequencing projects have since shown that insect chitinase-like sequences comprise a relatively large and diverse family of genes (Fig. 3). The number of chitinase-like genes present appears to be linked to a metamorphic lineage, with hemimetabolous species generally having fewer chitinase genes compared with holometabolous species. Based on phylogenetic analyses, expansion of the chitinase family in holometabolous species, in particular members of Diptera and Coleoptera, is likely the result of successive gene duplications. Nakabachi et al. (2010) suggested that the gene expansion might reflect the need for extensive remodeling of body structures during development in holometabolous species; thus, the need for a multitude of degradation enzymes that can be expressed in different tissues and developmental points. In support of this, chitinase-like genes often differ in size, expression profile, domain architecture, and functionality (Zhu et al., 2008a; Zhang et al., 2011; Zhang et al., 2012; Xi et al, 2015).

Currently, the insect chitinase-like family can be phylogenetically subdivided into eight groups or clades (Fig. 4), constituents of which often exhibit distinctly different developmental expression patterns and tissue specificity as well as differences in substrate specificity and biochemical properties. Based on RNAi-mediated knockdown studies (Zhu

et al., 2008b; Khajuria et al., 2010; Zhang et al., 2012; Li et al., 2015; Xi et al., 2015), group I and II genes appear to function in cuticular digestion, group III in abdominal contractions and wing expansion, and group IV in degradation of chitin in the peritrophic membrane that lines the lumen of the midgut. Among these groups the group I chitinases have been most extensively studied, with multiple genes, many of which appear to be the result of gene duplications, reported in *Anopheles gambiae* (five), *Aedes aegypti* (four), *Culex quinquefasciatus* (three), *Locusta migratoria* (two), *P. humanus* (two), and *N. lugens* (two). Similar to *L. hesperus*, the latter species is also a hemipteran pest, however, phylogenetic analysis of the LhChts clustered only one, LhCht1, with the group I chitinases. This could indicate that our inventory of LhChts is incomplete, which is possible since the transcriptomic data analyzed were generated from whole body adults and would thus be deficient in genes restricted to the nymphal stages. Alternatively, given that the complement of chitinase-like sequences we identified in our transcriptomic data are comparable to the genomic results reported for other hemipterans (Fig. 3), the lack of multiple group I chitinases could reflect differentiation between the two species. Indeed, only a single group I gene was identified in *A. pisum* (Nakabachi et al., 2010).

Because of their crucial role in insect growth and development, chitinases have been proposed as potential candidates for targeted disruption by chemical-based pesticides or, more recently, transgenic approaches. Indeed, the lethal phenotype (i.e., incomplete ecdysis) reported in a number of species following RNAi-based knockdown of various chitinases (Zhu et al., 2008b; Zhang et al., 2012; Li et al., 2015; Xi et al., 2015) supports this potential. Furthermore, Agrawal et al. (2015) recently reported cessation of *H. armigera* larval molting after feeding on transgenic tobacco engineered to express a miRNA targeting the *H. armigera* group I chitinase *hachi*. Future studies in *L. hesperus* will focus on using RNAi-based methods to elucidate the roles of the 10 chitinase-like sequences in molting and determining their potential usefulness in controlling populations of this pest species.

ACKNOWLEDGMENTS

The authors thank Daniel Langhorst for maintaining the *Lygus hesperus* colony and Lynn Forlow Jech for assistance with tissue dissections. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

LITERATURE CITED

Adler PN, Sobala LF, Thom D, Nagaraj. R. 2013. Dusky-like is required to maintain the integrity and planar cell polarity of hairs during the development of the *Drosophila* wing. Dev Biol 379:76–91.

Agrawal N, Sachdev B, Rodrigues J, Sree KS, Bhatnagar RK. 2013. Development associated profiling of chitinase and microRNA of *Helicoverpa armigera* identified chitinase repressive microRNA. Sci Rep 3:2292.

Agrawal A, Rajamani V, Reddy VS, Mukherjee SK, Bhatnagar RK. 2015. Transgenic plants overexpressing insect-specific microRNA acquire insecticidal activity against *Helicoverpa armigera*: an alternative to Bt-toxin technology. Transgenic Res 24:1–11.

Ahmad T, Rajagopal R, Bhatnagar RK. 2003. Molecular characterization of chitinase from polyphagous pest *Helicoverpa armigera*. Biochem Biophys Res Commun 310:188–195.

Arakane Y, Muthukrishnan S. 2010. Insect chitinase and chitinase-like proteins. Cell Mol Life Sci 67:201–216.

Brent CS. 2010. Stage-specific effects of population density on the development and fertility of the western tarnished plant bug, *Lygus hesperus*. J Insect Sci 10:59.

Brent CS, Peeters C, Dietmann V, Crewe R, Vargo E. 2006. Hormonal correlates of reproductive status in the queenless ponerine ant, *Streblognathus peetersi*. J Comp Physiol A 192:315–320.

Butler GD. 1970. Temperature and the development of egg and nymphal stages of *Lygus desertus*. J Econ Entomol 63:1994–1995.

Butler GD, Wardecker AL. 1971. Temperature and the development of eggs and nymphs of *Lygus hesperus*. Ann Entomol Soc Am 64:144–145.

Camp AA, Funk DH, Buchwalter DB. 2014. A stressful shortness of breath: molting disrupts breathing in the mayfly *Cloeon dipterum*. Freshwater Sci 33:695–699.

Champlain RA, Butler GD. 1967. Temperature effects on development of the egg and nymph stages of *Lygus hesperus* (Hemiptera: Miridae). Ann Entomol Soc Am 60:519–521.

Claros MG, von Heijne G. 1994. TopPred II: an improved software for membrane protein structure predictions. Comput Appl Biosci 10:685–686.

Cooper WR, Spurgeon WD. 2012. Temperature dependent development of *Lygus hesperus* (Hemiptera: Miridae) nymphs. J Econ Entomol 105:860–865.

Cooper WR, Spurgeon WD. 2013. Temperature-dependent egg development of *Lygus hesperus* (Hemiptera: Miridae). J Econ Entomol 106:124–130.

Cruz J, Nieva C, Mané-Padrós D, Martín D, Bellés X. 2008 Nuclear receptor BgFTZ-F1 regulates molting and the timing of ecdysteroid production during nymphal development in the hemimetabolous insect *Blattella germanica*. Dev Dyn 237:3179–3191.

Debolt JW. 1982. Meridic diet for rearing successive generations of *Lygus hesperus*. Ann Entomol Soc Am 75:119–122.

Ding X, Gopalakrishnan B, Johnson LB, White FF, Wang X, Morgan TD, Kramer KJ, Muthukrishnan S. 1998. Insect resistance of transgenic tobacco expressing an insect chitinase gene. Transgenic Res 7:77–84.

Doucet D, Retnakaran A. 2012. Insect chitin: metabolism, genomics and pest management. In: Dhadialla TS, editor. Advances in insect physiology: insect growth disruptors. Vol. 43. Burlington: Academic Press. p 438–511.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797.

Filho BPD, Lemos FJA, Secundino NFC, Páscoa V, Pereira ST, Pimenta, PFP. 2002. Presence of chitinase and beta-*N*-acetylglucosaminidase in the *Aedes aegypti* chitinolytic system involving peritrophic matrix formation and degradation. Insect Biochem Mol Biol 32:1723–1729.

Finn RD, Clements J, Eddy SR. 2011. HMMER web server: interactive sequence similarity searching. Nucleic Acids Res 39:W29–37.

Fukamizo T, Kramer KJ. 1985. Mechanism of chitin hydrolysis by the binary chitinase system in insect moulting fluid. Insect Biochem 15:141–145.

Fukamizo T, Kramer KJ. 1987. Effect of 20-hydroxyecdysone on chitinase and β-N-acetylglucosaminidase during the larval–pupal transformation of *Manduca sexta* (L.). Insect Biochem 17:547–550.

Henrissat B, Bairoach A. 1993. Updating the sequence-based classification of glycosyl hydrolases. Biochem J 316:695–696.

Hofmann K, Stoffel W. 1993. TMBASE – a database of membrane spanning protein segments. Biol Chem Hoppe-Seyler 374:166.

Hull JJ, Geib SM, Fabrick JA, Brent CS. 2013. Sequencing and *de novo* assembly of the western tarnished plant bug (*Lygus hesperus*) transcriptome. PLoS One 8:e55105.

Hull JJ, Chaney K, Geib SM, Fabrick JA, Brent CS, Walsh D, Lavine LC. 2014. Transcriptome-based identification of ABC transporters in the western tarnished plant bug *Lygus hesperus*. PLoS One 9:e113046.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649.

Khajuria C, Buschman LL, Chen M-S, Muthukrishnan S, Zhu KY. 2010. A gut-specific chitinase gene essential for regulation of chitin content of peritrophic matrix and growth of *Ostrinia nubilalis* larvae. Insect Biochem Mol Biol 40:621–629.

Kim MG, Shin SW, Bae KS, Kim SC, Park HY. 1998. Molecular cloning of chitinase cDNAs from the silkworm, *Bombyx mori* and the fall webworm, *Hyphantria cunea*. Insect Biochem Mol Biol 28:163–171.

Koga D, Funakoshi T, Fujimoto H, Kuwano EEM. 1991. Effects of 20-hydroxyecdysone and KK-42 on chitinase and beta-N-acetylglucosaminidase during the larval-pupal transformation of *Bombyx mori*. Insect Biochem 21:277–284.

Kramer KJ, Koga D. 1986. Insect chitin: physical state, synthesis, degradation and metabolic regulation. Insect Biochem 16:851–877.

Kramer KJ, Muthukrishnan S. 2005. Chitin metabolism in insects. In: Gilbert LI, Iatrou K, Gill S, editors. Comprehensive molecular insect science. Vol. 4, biochemistry and molecular biology. Oxford: Elsevier Press. p 111–144.

Kramer KJ, Corpuz L, Choi HK, Muthukrishnan S. 1993. Sequence of a cDNA and expression of the gene encoding epidermal and gut chitinases of *Manduca sexta*. Insect Biochem Mol Biol 23:691–701.

Kramer KJ, Muthukrishnan S, Johnson L, White F. 1996. Chitinases for insect control. In: Carozzi N, Koziel M, editors. Advances in insect control: transgenic plants for the control of insect pests. Washington, DC: Taylor and Francis. p 185–193.

Kubo I, Klocke JA, Asano S. 1981. Insect ecdysis inhibitors from the East African medicinal plant *Ajuga rernota* (Labiatae). Agric Biol Chem 45:1925–1927.

Lawrence SD, Novak NG. 2006. Expression of poplar chitinase in tomato leads to inhibition of development in Colorado potato beetle. Biotechnol Lett 28:593–599.

Li D, Zhang J, Wang Y, Liu X, Ma E, Sun Y, Li S, Zhu KY, Zhang J. 2015. Two chitinase 5 genes from *Locusta migratoria*: molecular characteristics and functional differentiation. Insect Biochem Mol Biol 58:46–54.

Lombard V, Ramulu HG, Drula E, Coutinho PM, Henrissat B. 2014. The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res 42:D490–D495.

Mané-Padrós D, Cruz J, Vilaplana L, Nieva C, Ureña E, Bellés X, Martín D. 2010. The hormonal pathway controlling cell death during metamorphosis in a hemimetabolous insect. Dev Biol 346:150–160.

Mané-Padrós D, Borràs-Castells F, Bellés X, Martín D. 2012. Nuclear receptor HR4 plays an essential role in the ecdysteroid-triggered gene cascade in the development of the hemimetabolous insect *Blattella germanica*. Mol Cell Endocrinol 348:322–330.

Merzendorfer H, Zimoch L. 2003. Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. J Exp Biol 206:4393–4412.

Nakabachi A, Shigenobu S, Miyagishima S. 2010. Chitinase-like proteins encoded in the genome of the pea aphid, *Acyrthosiphon pisum*. Insect Mol Biol 19:175–185.

Pan Y, Lü P, Wang Y, Yin L, Ma H, Ma G, Chen K, He Y. 2012. In silico identification of novel chitinase-like proteins in the silkworm, *Bombyx mori*, genome. J Insect Sci 12:150.

Patana R. 1982. Disposable diet packet for feeding and oviposition of *Lygus hesperus* (Hemiptera: Miridae). J Econ Entomol 75:668–669.

Petersen TN, Brunak S, Heijne GV, Nielsen H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8:785–786.

Regev A, Keller M, Strizhov M, Sneh B, Prudovsky E, Chet I, Ginzberg I, Koncz Z, Schell J, Zilberstein A. 1996. Synergistic activity of a *Bacillus thuringinensis* d-endotoxin and bacterial endochitinase against *Spodoptera littoralis* larvae. Appl Environ Microbiol 62:3581–3586.

Riddiford LM, Hiruma K, Zhou X, Nelson CA. 2003. Insights into the molecular basis of the hormonal control of molting and metamorphosis from *Manduca sexta* and *Drosophila melanogaster*. Insect Biochem Mol Biol 33:1327–1338.

Royer V, Fraichard S, Bouhin H. 2002. A novel putative insect chitinase with multiple catalytic domains: hormonal regulation during metamorphosis. Biochem J 366:921–928.

Schuh RT. 2013. On-line systematic catalog of plant bugs (Insecta: Heteroptera: Miridae). Available at http://research.amnh.org/pbi/catalog/.

Schwartz MD, Foottit RG. 1998. Revision of the nearctic species of Lygus Hahn, with a review of the palearctice species (Heteroptera: Miridae). In: Gupta VK, editor. Memoirs on Entomology, International. Vol. 10. Associated Publishers, Gainesville, FL. p 428.

Scott DR. 1977. An annotated listing of host plants of *Lygus hesperus* Knight. Entomol Soc Am Bull 23:19–22.

Shinoda T, Kobayashi J, Matsui M, Chinzei Y. 2001. Cloning and functional expression of a chitinase cDNA from the common cutworm, *Spodoptera litura*, using a recombinant baculovirus lacking the virus-encoded chitinase gene. Insect Biochem Mol Biol 31:521–532.

Snodgrass GL. 1996. Insecticide resistance in field populations of the tarnished plant bug (Heteroptera: Miridae) in cotton in the Mississippi Delta. J Econ Entomol 89:783–790.

Snodgrass G, Scott W. 2002. Tolerance to acephate in tarnished plant bug (Heteroptera: Miridae) populations in the Mississippi river delta. Southwest Entomol 27:191–199.

Snodgrass G, Gore J, Abel C, Jackson R. 2009. Acephate resistance in populations of the tarnished plant bug (Heteroptera: Miridae) from the Mississippi River Delta. J Econ Entomol 102:699–707.

Sun LJ, Liu YJ, Shen CP. 2015. The effects of exogenous 20-hydroxyecdysone on the feeding, development, and reproduction of *Plutella xylostella* (Lepidoptera: Plutellidae). Florida Entomol 98:606–612.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30:2725–2729.

Tanaka Y, Naya S. 1995. Dietary effect of ecdysone and 20-hydroxyecdysone on larval development of two lepidopteran species. Appl Entomol Zool 30:285–294.

Truman JW. 2005. Hormonal control of insect ecdysis: endocrine cascades for coordinating behavior with physiology. Vitam Horm 73:1–30.

von Heijne G. 1992. Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. J Mol Biol 225:487–494.

Wang HB, Ali SM, Moriyama M, Iwanaga M, Kawasaki H. 2012. 20-Hydroxyecdysone and juvenile hormone analog prevent precocious metamorphosis in recessive trimolter mutants of *Bombyx mori*. Insect Biochem Mol Biol 42:102–108.

Warren JT, Gilbert LI. 1986. Ecdysone metabolism and distribution during the pupal-adult development of *Manduca sexta*. Insect Biochem 16:65–82.

Warren JT, Smith W, Gilbert LI. 1984. Simplification of the ecdysteroid radioimmunoassay by the use of protein A from *Staphylococcus aureus*. Experientia 40:393–394.

Wheeler AG. 2001. Biology of the plant bugs (Hemiptera: Miridae): pests, predators, opportunists. New York: Comstock Publishing Associates. p 528.

Xi Y, Pan PL, Ye YX, Yu B, Xu HJ, Zhang CX. 2015. Chitinase-like gene family in the brown planthopper, *Nilaparvata lugens*. Insect Mol Biol 24:29–40.

Yang WJ, Xu KK, Zhang RY, Dou W, Wang JJ. 2013. Insights into the molecular basis of the hormonal control of molting and metamorphosis from *Manduca sexta* and *Drosophila melanogaster*. Int J Mol Sci 14:20048–20063.

Zhang J, Zhang X, Arakane Y, Muthukrishnan S, Kramer KJ, Ma E, Zhu KY. 2011. Comparative genomic analysis of chitinase and chitinase-like genes in the African malaria mosquito (*Anopheles gambiae*). PLoS One 6:e19899.

Zhang D, Chen J, Yao Q, Pan Z, Chen J, Zhang W. 2012. Functional analysis of two chitinase genes during the pupation and eclosion stages of the beet armyworm *Spodoptera exigua* by RNA interference. Arch Insect Biochem Physiol 79:220–234.

Zheng Y, Zheng S, Cheng X, Ladd T, Lingohr EJ, Krell PJ, Arif BM, Retnakaran A, Feng Q. 2002. A molt-associated chitinase cDNA from the spruce budworm, *Choristoneura fumiferana*. Insect Biochem Mol Biol 32:1813–1823.

Zheng YP, Retnakaran A, Krell PJ, Arif BM, Primavera M, Feng QL. 2003. Temporal, spatial and induced expression of chitinase in the spruce budworm, *Choristoneura fumiferana*. J Insect Physiol 49:241–247.

Zhu Q, Maher EA, Masoud S, Dixon RA, Lamb CJ. 1994. Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. Nat Biotechnol 12:807–812.

Zhu Q, Deng Y, Vanka P, Brown SJ, Muthukrishnan S, Kramer KJ. 2004. Computational identification of novel chitinase-like proteins in the *Drosophila melanogaster* genome. Bioinformatics 20:161–169.

Zhu Q, Arakane Y, Banerjee D, Beeman RW, Kramer KJ, Muthukrishnan S. 2008a. Domain organization and phylogenetic analysis of the chitinase-like family of proteins in three species of insects. Insect Biochem Mol Biol 38:452–466.

Zhu Q, Arakane Y, Beeman RW, Kramer KJ, Muthukrishnan S. 2008b. Functional specialization among insect chitinase family genes revealed by RNA interference. Proc Natl Acad Sci USA 105:6650–6655.

Zitnan D, Adams ME. 2005. Neuroendocrine regulation of insect ecdysis. In: Gilbert LI, Iatrou K, Gill SS, editors. Comprehensive insect biochemistry, physiology, pharmacology, and molecular biology. Vol 3. London: Elsevier Press. p. 1–60.