Insect Molecular Biology

Insect Molecular Biology (2010) **19**(6), 787–798 doi: 10.1111/j.1365-2583.2010.01035.x

# Molecular cloning, transcriptional regulation, and differential expression profiling of vitellogenin in two wing-morphs of the brown planthopper, *Nilaparvata lugens* Stål (Hemiptera: Delphacidae)

**M. Tufail*‡, M. Naeemullah†‡, M. Elmogy*, P. N. Sharma†, M. Takeda* and C. Nakamura†**

**Insect Science Lab., Graduate School of Agricultural Science, Kobe University, Nada, Kobe, Japan; and †Plant Genetics Lab., Graduate School of Agricultural Science, Kobe University, Nada, Kobe, Japan*

## Abstract

**The brown planthopper, *Nilaparvata lugens*, is a serious pest of rice crops throughout Asia and exhibits wing dimorphism, with brachypterous adults having reduced wings and macropterous adults possessing fully developed wings. To understand the reproductive strategies in two wing-morphs of this insect, the transcript encoding the major yolk protein precursor, vitellogenin (Vg), was cloned. The complete mRNA transcript was 6314 bp, which encodes a protein of 2063 residues including an 18-residue putative signal peptide. Analysis of the mature protein revealed two vitellogenin-N (or lipoprotein amino-terminal) domains near the N-terminus and a von Willebrand factor type D domain near the C-terminus. In addition, a highly conserved motif GL/ICG, and a number of cysteine residues were identified near the C-terminus. Northern blot analysis identified a ~6.8 kb *Vg* gene transcript that was expressed exclusively in the adult female fat body cells. The expression profile revealed that the Vg gene starts to be expressed earlier (on day 3) in brachypters as compared to macropters where the mRNA transcript was observed on day 4. However, in both morphs, the amount of Vg mRNA increased to reach high levels during vitellogenic periods [from day 4 (in brachypters) and day 5 (in macropters) and onwards]. Reflecting the RNA transcription pattern, the Vg signal was detected by immunoblotting on day 3 and day 4 in haemolymph of brachypterous and macropterous females, respectively, and that was increased every day and remained high during the vitellogenic periods. Furthermore, the topical application of juvenile hormone (JH) III had up-regulated the *Vg* gene expression suggesting that the *Vg* gene is regulated by JH in *N. lugens*. In addition, it was demonstrated by Southern blot analysis that there exists a single copy of the gene in the *N. lugens* genome. A delayed trend in expression (of both the transcript and the protein) demonstrated by macropterous females in the present studies supports the hypothesis of prereproductive long distance migration in this wing-dimorphic species.**

**Keywords: brown planthopper, *Nilaparvata lugens* Stål, vitellogenin, cDNA, mRNA, expression, regulation.**

## Introduction

The brown planthopper, *Nilaparvata lugens* (Nl) is one of the most destructive insect pests of rice in many rice-growing areas of Asia and Oceania. This species has two wing morphs, brachypterous (nonmigratory) and macropterous (migratory), and the latter invades Japan annually from southern China during rice seasons with the assistance of the monsoon winds where it reproduces tremendously and causes considerable economic losses. Many reports on migration of *N. lugens* are available (Kisimoto, 1976; Cheng *et al.*, 1979; Sogawa & Watanabe, 1992); however, information regarding the molecular properties of its reproduction is lacking. Thus, characterization of the gene encoding the major yolk protein precursor, vitellogenin (Vg), would be helpful to understand the reproduction strategies in this species and may allow the development of some pest control

First published online 4 August 2010.

Correspondence: Muhammad Tufail, Graduate School of Agricultural Science, Kobe University, Nada, Kobe 657-8501, Japan. Tel./fax: +81 78 803 5869; e-mail: mtufail@kobe-u.ac.jp; tufailm2001@yahoo.com

‡The first two authors contributed equally to this work.

schemes. The reproductive success of all oviparous species including insects depends on Vg biosynthesis and its accumulation in the developing oocytes. During the reproductive phase, the precursor Vg is synthesized in large amounts in the female fat body, secreted into haemolymph, and then taken up by the developing oocytes by receptor-mediated endocytosis (Byrne *et al*., 1989; Raikhel & Dhadialla, 1992; Hagedorn *et al*., 1998; Sappington & Raikhel, 1998; Snigirevskaya & Raikhel, 2005; Tufail & Takeda, 2009). Once sequestered, the Vgs are stored in a crystalline form as vitellins (Vns), a nutrient source for the developing embryos (Kunkel & Nordin, 1985).

Owing to the primary role of Vgs in reproduction, their primary structures have been sequenced from a wide group of animals, both vertebrates and invertebrates. In insects, a *Vg* gene transcript of 6–7 kb encodes a primary protein of ~200 kDa, which is enzymatically cleaved into smaller subunit polypeptides before being secreted into the haemolymph (see reviews: Sappington & Raikhel, 1998; Tufail *et al*., 2005; Tufail & Takeda, 2008). This cleavage is performed by the subtilisin-like endoproteases, convertases (Barr, 1991; Rouille *et al*., 1995) following a consensus sequence motif R/KXXR/K. The first experimental demonstration of subtilisin-like convertase involvement in pro-Vg cleavage was reported by Chen & Raikhel (1996). Until now, Vg primary structures have been reported from several insect species (see a recent review by Tufail & Takeda, 2008 for references). Vgs are members of a larger superfamily of molecules known as large lipid transfer proteins (Babin *et al*., 1999). Despite their low overall conservation across major taxonomic groups, their common function and disproportionate conservation of certain amino acids suggest a common phylogenetic ancestor (Chen *et al*., 1997; Sappington *et al*., 2002).

In insects showing wing dimorphism, it is suggested that there are fitness costs associated with the ability to fly (Roff, 1984, Denno *et al*., 1991). The energy used to construct wings and flight muscles is simply not available for reproductive investment (Zera & Denno, 1997). When raised on the same source, macropterous females have lower fecundity than flightless females in crickets (Roff, 1984) and planthoppers (Kisimoto, 1965, Denno *et al*., 1989). Additionally, reproduction is delayed in the migratory forms of many female insects including *N. lugens* (Roff, 1986; Ayoade *et al*., 1999). In *N. lugens*, previous studies provide information on ovarian development/number of eggs (when reared on a resistant rice variety 'Mudgo') (Sogawa & Pathak, 1970), on oocyte growth by applying juvenile hormones (JHs) or methoprene and identifying the variation of the pre-ovipositional period in two-wing morphs (Iwanaga & Tojo, 1986; Ayoade *et al*., 1999) and on oviposition behaviour (Hattori & Sogawa, 2002). Furthermore, investigations in our labs have revealed the oviposition rate by rearing of nonvirulent *N. lugens* biotypes on rice varieties/cultivars carrying *N. lugens* resistance genes (Ketipearachchi *et al*., 1998; Naeemullah *et al*., 2009). Nevertheless, there is no knowledge of the structure and regulation of the major yolk protein precursor, Vg, in the two wing-forms of this pest. To our knowledge, the only report on NlVg available is by Cheng & Hou (2005) but it is restricted to only Vg identification and its cellular distribution in this insect. Thus, to understand the reproduction strategies in two wing morphs of *N. lugens* at the molecular level, we cloned a complete cDNA encoding Vg, and show here how expression timing of both the transcript and the protein differs in nonmigratory and migratory females. We also investigated the regulation of NlVg transcription by JH III, and showed that there exists only a single copy of the *Vg* gene in this insect. Next, we identified, on a molecular basis, a 50-kDa Vg polypeptide in addition to the 175-kDa polypeptide previously identified through immunoblotting by Cheng & Hou (2005). In addition, NlVg was compared with other insect and non-insect Vgs and a molecular phylogenetic tree constructed.

## Results and discussion

### *Cloning and structural analysis of NlVg*

Using a rapid amplification of cDNA ends PCR (RACE-PCR) strategy, we cloned the complete cDNA encoding Vg of *N. lugens*. The complete sequence obtained by the contig alignment of the three RACE-PCR clones (see Experimental procedures) was 6314 bp, consisting of a 12 bp 5′-untranslated region (UTR), an open reading frame of 2063 residues and a 110-nucleotide 3′-UTR (accession number: AB353856 and Fig. 1). The analysis of the primary protein revealed that the first 18 amino acids correspond to a putative signal peptide [as analysed with the SignalP program (http://www.cbs.dtu.dk/services/SignalP/)] (Fig. 1). The predicted molecular weight of the mature protein was 227.94 kDa with an isoelectric point (pI) of 8.69. The National Center for Biotechnology Information (NCBI) Conserved Domain Database (CCD) search (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) predicted two vitellogenin-N domains (also known as the lipoprotein N-terminal regions) at the amino-terminus of the NlVg (amino acid positions: 24–339 and 584–932, respectively). These domains are implicated in lipid transfer activity (see review: Smolenaars *et al*., 2007). A third domain, the von Willebrand factor type D (VWD) domain, was identified at the C-terminus (amino acid position: 1767–1884). In addition, three polyserine regions, three RXXR consensus cleavage sites, the GL/ICG motif, and the cysteine residues conserved at the C-terminus were identified

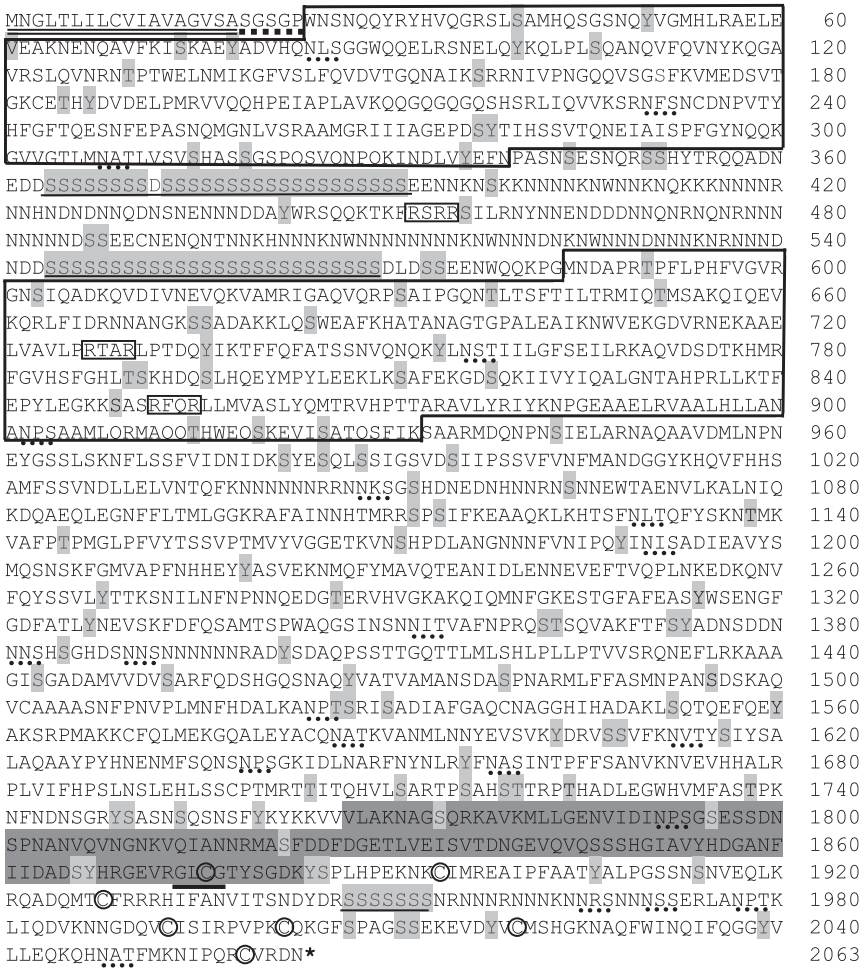

**Figure 1.** Deduced amino acid sequence of the *Nilaparvata lugens* vitellogenin (NlVg) protein and its modular architecture. Amino acids are numbered on the right from the initiation methionine. The two vitellogenin_N domains delimited by the National Center for Biotechnology Information Conserved Domain Search are indicated with boxes at the N-terminus, whereas the von Willebrand factor domain is shown by a dark-shaded box at the C-terminus. The regions rich in polyserine tracts are underlined. Three consensus RXXR sequence motifs for possible cleavage sites are boxed. The putative glycosylation sites (NXS/T) are dotted-underlined, whereas the putative phosphorylated serine (S), threonine (T) and tyrosine (Y) residues (predicted using the NetPhos 2.0 program) are shown with light-shaded frames. The putative signal peptide (predicted using the SignalP program) is double-underlined. The GL/ICG motif is shown with a bold line. The chemically determined N-terminal amino acid sequence for the 50 kDa vitellin (Vn) polypeptide is underlined with a bold dotted line. The asterisk indicates the stop codon. This sequence has been submitted to GenBank and assigned the accession number: AB353856.

(Fig. 1). Additionally, unique poly-N runs, encompassed by usual polyserine domains, have been observed at the N-terminus of the NlVg. The existence of these poly-N runs in NlVg is striking and unusual; however, whether they play any physiological role in this insect remains unclear. Furthermore, 20 putative glycosylation sites (NXS/T) and 181 potential phosphorylation residues were found in the NlVg sequence (Fig. 1). The presence of these modified residues in NlVg indicates that this molecule is likely to be highly phosphorylated. The phosphate moieties have a negative charge and may play a role in receptor binding as proposed by Sappington *et al*. (1996).

The NlVg sequence was compared with those of other known insect Vgs. The NlVg had, in general, high amino acid identity (23–35%) within the hemimetabolous insects as compared to holometabolous insects, where the identity ratio was 20–30%. The exception was that of *Athalia rosae*, where the identity ratio was 36%. Indeed, the highest amino acid identity (30–35%) was found within the members of the order Hemiptera. The comparison of NlVg with other insect Vgs shows a high degree of conservation; the motif GL/ICG and the polycysteines at conserved locations near the C-terminus (see Tufail & Takeda, 2008) (Fig. 1) are particularly interesting. Recently, Akasaka *et al*. (2010) have reported that the C-terminal region and VWD domain of vitellogenin are processed and attached to the vitelline coat, which in turn participate in fertilization as the binding partners of sperm proteases.

*Phylogenetic relationship with Vgs of other arthropods and vertebrates*

To clarify the evolutionary relationship of NlVg, we used a neighbor-joining tree construction method based on distances of 60 Vg sequences (excluding the signal peptide) from 46 arthropod and non-arthropod species. The dendrogram obtained places the NlVg with the other insects and particularly with hemipterans as a distinct cluster (Fig. 2). Moreover, the tree clusters different insect species into specific orders in a quite coherent manner reflecting that there exists a greater sequence similarity within the group than beyond the group level. The tree also clearly divides the Vgs from insects, arachnids, vertebrates and crustaceans into separate groups as indicated by their known phylogeny. Nevertheless, the nematode Vgs appear as a sister group of arachnid Vgs. The present phylogenetic analysis reveals that insect Vgs are more close to arachnid and nematode Vgs than to vertebrate and crustacean Vgs. The crustacean Vgs form a very external strongly supported cluster (100% bootstrap value) and thus remain the most divergent from all other Vgs.

*Genomic copy number of the* NlVg *gene*

To investigate the copy number of the *Vg* gene, Southern analysis was carried out with 10 μg genomic DNA and a Vg cDNA probe synthesized from the 5′-end part of the NlVg cDNA (nt position: 1–1001) as a probe. The hybridization signal revealed a single discrete band when digested with *Eco*RV, *Not*I and *Xho*I (Fig. 3), suggesting that a single copy of the *Vg* gene exists in the genome of *N. lugens*.

*Expression pattern and temporal profile of* Vg *gene transcription in two wing-morphs of* N. lugens

To probe the sex-, tissue- and stage-specific expression, and the temporal profile of NlVg gene expression, we performed a Northern blot analysis with total RNA extracted from the adult males and females, and from the nymph whole bodies (Fig. 4). The hybridization analysis detected a single transcript of ~6.8 kb exclusively in the fat body of adult-females as we expected (Fig. 4A). No trace of hybridization signal was detected with total RNA from adult males or from nymphs. The *Vg* gene transcript of 6–7 kb has been identified from several insect species (see review: Tufail & Takeda, 2008). The developmental profile of *Vg* gene transcription in brachypterous females reveals that it starts expressing on day 3 of adult emergence, and continues to increase every day to reach its maximum on day 7, and then starts to decline (Fig. 4B, C). By contrast, the *Vg* gene transcript was observed one day later, on day 4, in the macropterous females (Fig. 4B, C) although Vg mRNA levels were seen to be high, similar to their brachypterous counterparts, during the vitellogenic period from day 5 onwards (Fig. 4B, C).

The discrepancy in reproductive strategies has been reported in several insects exhibiting wing polymorphism including *N. lugens* (Kisimoto, 1965; Fujisaki, 1986). In *N. lugens*, the difference in pre-oviposition periods in two wing-forms has been reported (Ohkubo, 1967), and the general trend demonstrated was that the macropterous females considerably delayed the initiation of oviposition compared to brachypterous females under the same rearing conditions (Kisimoto, 1965; Wada *et al*., 2007). In *Gryllus rubens*, greater ovarian growth was reported in the short-winged form than the long-winged morphs (Zera & Rankin, 1989). Moreover, in *N. lugens*, the earlier development of oocytes in brachypters was related to high JH titres in brachypters than those of macropters (Bertuso & Tojo, 2002). The different trait of JH titres in wing-dimorphic species is in keeping with the migratory syndrome influenced by the JH (Fairbairn, 1994) and the apparent flight capability vs. fecundity trade-off (Harrison, 1980). Our present findings of delayed expression of Vg mRNA in long-winged morphs probably demonstrate the longer developmental immaturity in these females that corresponds to the period of long distance migration.

*Identification, post-transcriptional processing and temporal expression profile of NlVgs*

The NlVgs were identified by comparing the polypeptide profiles of adult male and female whole body homogenates, haemolymph and of egg extracts. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Fig. 5A) revealed two Vg polypeptides (lane 3 and 5) of ~175 and ~50 kDa corresponding to the two Vn polypeptides (lane 4), whereas these polypeptides were absent in the male sample (lane 2). Our results differ from those reported by Cheng & Hou (2005) where only a single protein band of 175 kDa was reported. A possible explanation for this apparent discrepancy may be that we used 15% SDS-PAGE in our experiments for adequate resolution of the polypeptides instead of the 7.5% used by Cheng & Hou (2005). To further clarify whether the 50 kDa polypeptide is a post-transcriptionally cleaved product, we performed an amino-terminal analysis. The N-terminal sequence of the 50 kDa Vn polypeptide was SGSGP and located downstream of the putative signal peptide (Fig. 1), demonstrating, on a molecular basis, that the Vg of *N. lugens* also possesses a 50 kDa in addition to the 175 kDa polypeptide previously identified through immunoblotting by Cheng & Hou (2005) (Fig. 5A). The NlVg precursor polypeptide is most probably cleaved following RSRR cleavage signal (aa position: 452–455; Fig. 1) as

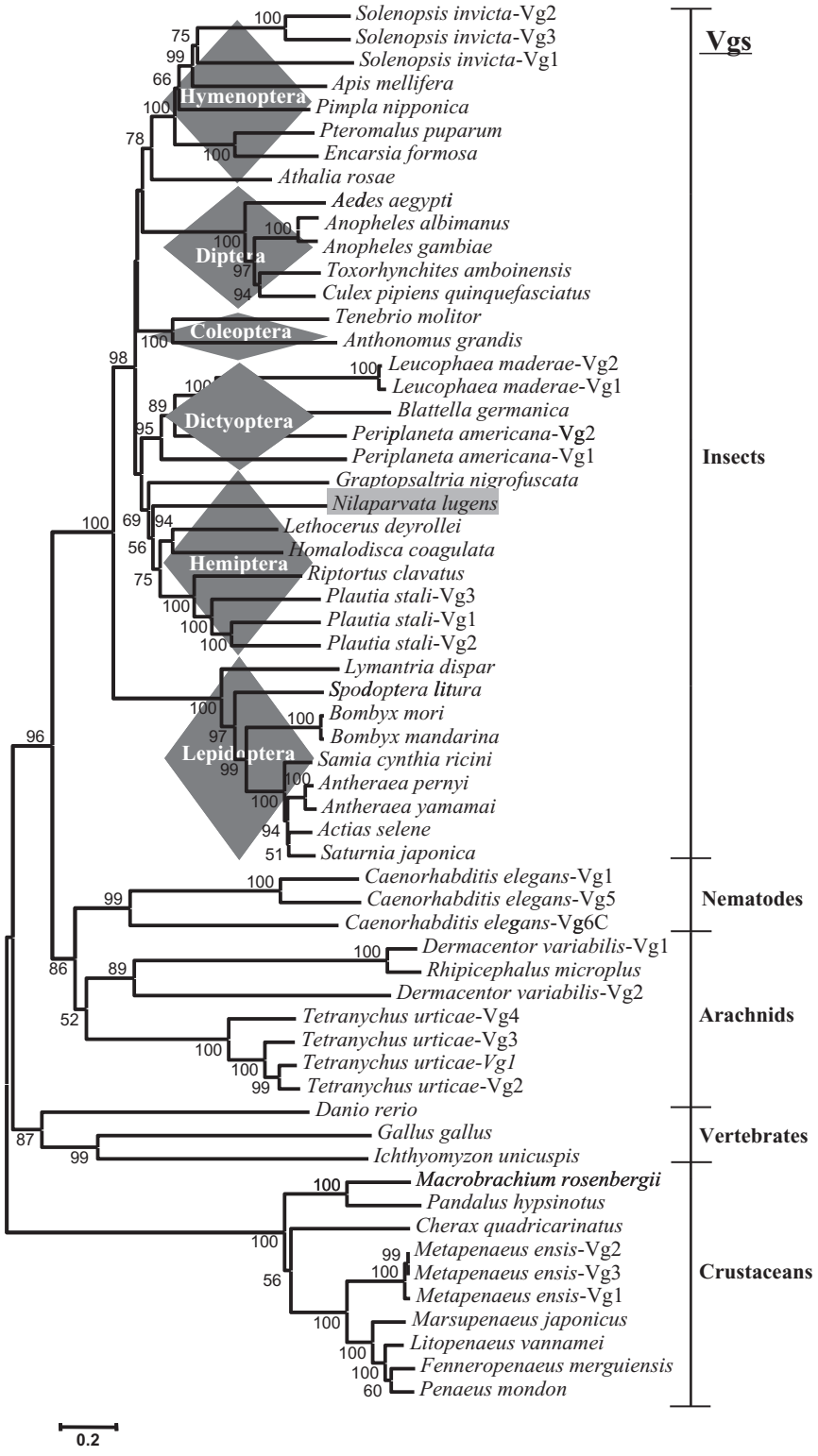


**Figure 2.** A molecular phylogenetic tree of the 60 insect and non-insect vitellogenins (Vgs). A distance analysis of amino acid sequences was performed using the CLUSTALW program (Thompson *et al*., 1994) and used as input for a neighbor-joining tree construction program (MEGA3, Kumar *et al*., 2001). The reference bar indicates the distance (number of amino acid substitutions per site). Bootstrap values (500 replications) are indicated at each node. Only support values >50% are shown. For sequence information see Experimental procedures.

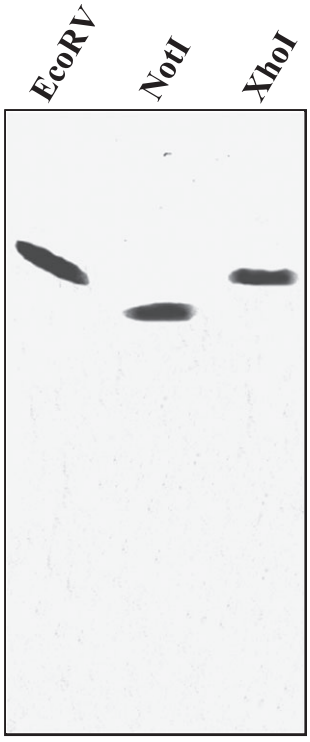


**Figure 3.** Analysis of genomic copy number of the *Nilaparvata lugens vitellogenin* (*NlVg*) gene. Southern blot analysis was performed with 10 μg genomic DNA digested with the restriction enzymes *Eco*RV, *Not*I and *Xho*I and separated on 1% gel. Blots were hybridized with an alkaline phosphatase-labelled NlVg cDNA fragment (see Experimental procedures).

the molecular weights predicted based on the amino acid sequences of small (48.33 kDa) and large (179.24 kDa) subunits correspond well with those identified by SDS-PAGE (Fig. 5A). The proteolytic cleavage of NlVg should not be surprising. The Vg of another hemipteran *Graptopsaltria nigrofuscata* was also cleaved into one large (200 kDa) and small (43 kDa) polypeptide (Lee *et al*., 2000). Recently, we have also determined enzymatic cleavage of Vg into two subunits in the water bug, *Lethocerus deyrollei* (Nagaba *et al*., 2010).

Based on the recent molecular studies, the consensus that has emerged is that the Vg molecule is cleaved (following the RXXR sequence motif) in almost all non-apocritan insect species, and this cleavage is found to be conserved at the N-terminus, mostly surrounded by polyserine stretches (Fig. 1), except for the *Lymantria dispar* Vg, in which the cleavage signal (RXXR) exists at the C-terminus (see reviews: Sappington & Raikhel, 1998; Tufail *et al*., 2005, Tufail & Takeda, 2008). Nevertheless, in Hemimetabola members (such as cockroaches and the bean bug, *Riptortus clavatus*) where the pro-Vg is cleaved into several subunits, additional RXXR cleavage motifs residing near the C-terminus or at the centre of the primary product are utilized (Hirai *et al*., 1998; Tufail & Takeda, 2002, 2008). However, the reason why Vgs are differentially cleaved in different insect species remains unclear.

To determine the developmental expression profile of Vg in both wing-morphs, haemolymph and egg extracts were separated on SDS-PAGE and assayed using immunoblot analysis (Fig. 5B). The antibody used was a generous gift from Dr Roger F. Hou and generated against the 175 kDa Vg peptide. The immunoblot analysis demonstrated the differential expression timing of Vg similar to the transcript in both wing-forms; the Vg signal was observed on day 3 in brachypters as compared to macropters where it first appeared on day 4 (Fig. 5B). The developmental expression profile revealed that Vg levels increase every day, reach a maximum on day 7, and then start to decrease in both wing morphs (Fig. 5B, C), suggesting sequestration by the developing oocytes. A similar pattern of Vg expression was observed in the other hemipteran, *R. clavatus*; however, the protein was detected one day after the Vg mRNA first appeared (Shinoda *et al*., 1996; Hirai *et al*., 1998), which is in contrast to NlVg where this protein was detected on the same day (day 3 in brachypters and day 4 in macropters) that Vg mRNA appeared (Fig. 4B). This is, perhaps, because of the short life of the adult *N. lugens* (only a few weeks) that Vg protein is expressed soon after the Vg gene is transcribed. The postponed expression of Vg protein and mRNA (Fig. 4B) demonstrated by macropters of *N. lugens* in our experiments supports the fitness cost hypothesis for long distance migration of this wing-dimorphic species. Studies on insect migration by flight have indicated that migration by females often occurs when they are sexually immature adults (Johnson, 1969). Hence, the pre-ovipositional period, the duration of the interval between adult emergence and reproductive maturity, often corresponds to the time during which the insect can express its migratory potential (Hill & Gatehouse, 1993; Wilson & Gatehouse, 1993; Wada *et al*., 2007; Zhao *et al*., 2009). In *N. lugens*, macropterous females also migrate prereproductively because most immigrants caught in the net traps (Kyushu, Japan) were unmated individuals with immature ovaries (Kisimoto, 1976). Thus, the delayed expression of both the transcript and the protein in macropterous females of *N. lugens* seems to be a good index of migration competence in this wing-dimorphic species.

### *Transcriptional regulation of the* NlVg *gene*

In order to evaluate the effect of exogenous JH on *NlVg* gene transcription, JH III was applied in a single dose (100 ng) on day 2 of female emergence with acetone as a control. Figure 6 shows that application of JH III induced significant expression of NlVg mRNA compared with untreated control or acetone-treated control samples. There is evidence that JH is responsible for oocyte matu-

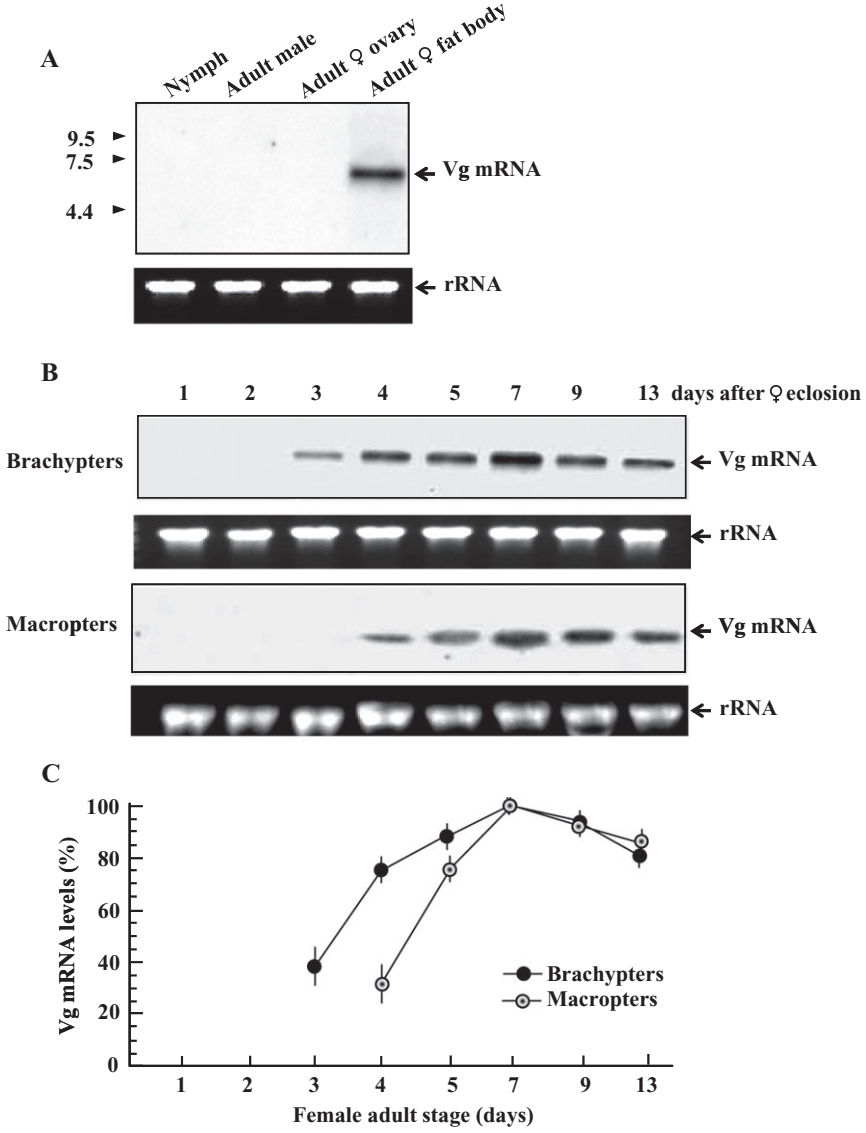


**Figure 4.** Expression pattern and developmental profile of the *Nilaparvata lugens vitellogenin* (*NlVg*) gene. (A) Northern blot hybridization analysis was performed with 20 μg total RNA extracted from the nymphs, adult male whole bodies and from adult female ovary and fat body cells. The RNA samples were probed with an alkaline phosphatase-labelled NlVg cDNA fragment (see Experimental procedures). The mobility of a standard size RNA marker (kb) is shown on the left. (B) NlVg mRNA expression profile in the adult brachypterous and macropterous females at the different developmental periods. Total RNA (20 μg) was used to detect the hybridization signal as mentioned above. Ribosomal RNAs (bottom panels) are shown as internal control after staining with ethidium bromide. (C) Quantification of NlVg mRNA levels from the Northern blots ($n = 3$) shown in (B) at different time periods. The day 7 Vg mRNA levels are expressed as 100%. The points show the average values of three independent experiments ± SE.

ration in *N. lugens* (Iwanaga & Tojo, 1986; Ayoade *et al*., 1999). Oogenesis also requires JH in other hemipteran species such as *Oncopeltus fasciatus* (Johansson, 1954), *Dysdercus intermedius* (Dittmann *et al*., 1985), *Spilostethus pandurus* (Ibanez *et al*., 1987), *R. clavatus* (Shinoda *et al*., 1996) and *Perillus bioculatus* (Adams *et al*., 2002). Moreover, the JH III was identified as the only type of JH in both nymphal and adult stages of *N. lugens* (Bertuso & Tojo, 2002). Thus, our results along with the previous studies suggest that JH may be involved in regulation of NlVg gene transcription in this species.

## Experimental procedures

### *Insects*

The *N. lugens* culture used in this study was obtained from the National Agricultural Research Centre for Kyushu Okinawa Region, Kumamoto, Japan. The founder population was originally collected from Nagasaki during cropping seasons of 1999 and is designated as Nagasaki-99. The culture had been maintained under laboratory conditions through sister-brother mating on a *japonica* rice cultivar 'Reiho', and then established on rice seedlings of a cultivar 'Nipponbare' in the Plant Genetics Laboratory, Faculty of Agriculture, Kobe University at 25 ± 0.5 °C under

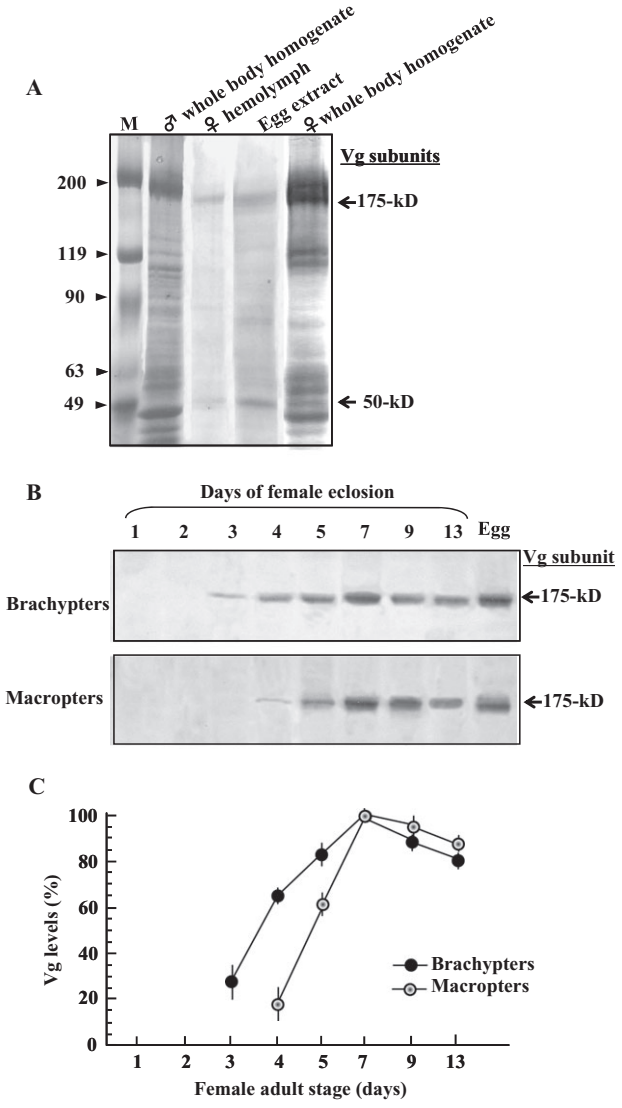


**Figure 5.** Identification and developmental profile of *Nilaparvata lugens* vitellogenins (NlVgs). (A) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of Vgs/vitellins (Vns) from adult male and female whole body homogenates, and from ahemolymph and egg extracts of *N. lugens.* The SDS-PAGE gel was stained with Coomassie blue. M and arrowheads on the left indicate the molecular weight markers (kDa). The identified Vns/Vgs are indicated by arrows on the right. (B) Developmental profile of 175 kDa Vg polypeptide in two wing morphs of *N. lugens* assayed by immunoblot analysis. Haemolymph samples (10 μg/lane) prepared from adult brachypterous and macropterous females at indicated periods were separated by SDS-PAGE, blotted to the polyvinylidene fluoride membrane and assayed by immunoblotting. Arrows on the right indicate the probed NlVgs. (C) Quantification of NlVg levels from the immunoblots shown in (B) at different time periods. The day 7 Vg protein levels are expressed as 100%. The points show the average values of three independent experiments ± SE.

16:8 h light : dark conditions with illumination by inflorescence lamps at an intensity of 110–120 μmol photons/$m^2$/s.

*Cloning of* NlVg *cDNA*

*NlVg* cDNA was cloned by the 3′- and 5′-RACE-PCR method using a Marathon cDNA amplification kit (Clontech, Mountain View, CA, USA) as reported previously (Tufail & Takeda, 2002). To amplify the 3′-end part of NlVg, the cDNA prepared from adult female whole bodies was used as a template with the degenerate primers and the adaptor primer 1 (AP1) (marathon cDNA adaptor, Clontech). The degenerate primers designed were based on the GL/ICG sequence motif (Tufail & Takeda, 2002), and were: 5′-GGA CTC TGT GG-3′, 5′-GGT CTG TGT GG-3′, 5′-GGT CTC TGC GG-3′, 5′-GGA CTG TGT GG-3′, 5′-GGT CTC TGT GG-3′, 5′-GGG CTC TGC GG-3′, 5′-GG(ATG) CT(GC) TG(CT) GG-3′, 5′-GGT ATT TGC GG-3′, 5′-GGC ATC TGT GG-3′ and 5′-GG(CT) AT(CT) TG(CT) GG-3′. PCR conditions employed were heating to 94 °C for 1 min, followed by 32 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 2 min. 5′-RACE-PCR was performed using a gene-specific primer 1 (GSP1) (nucleotide position: 5692–5716, GenBank accession number: AB353856) designed based on the initial 3′-end sequence and an AP1 (Clontech). The PCR conditions used were: 94 °C for 1 min, followed by 36 cycles of 94 °C for 5 s and 68 °C for 8 min. A second 5′-RACE-PCR was performed with GSP2 (nucleotide position: 1054–1078, GenBank accession number: AB353856) synthesized based on the first

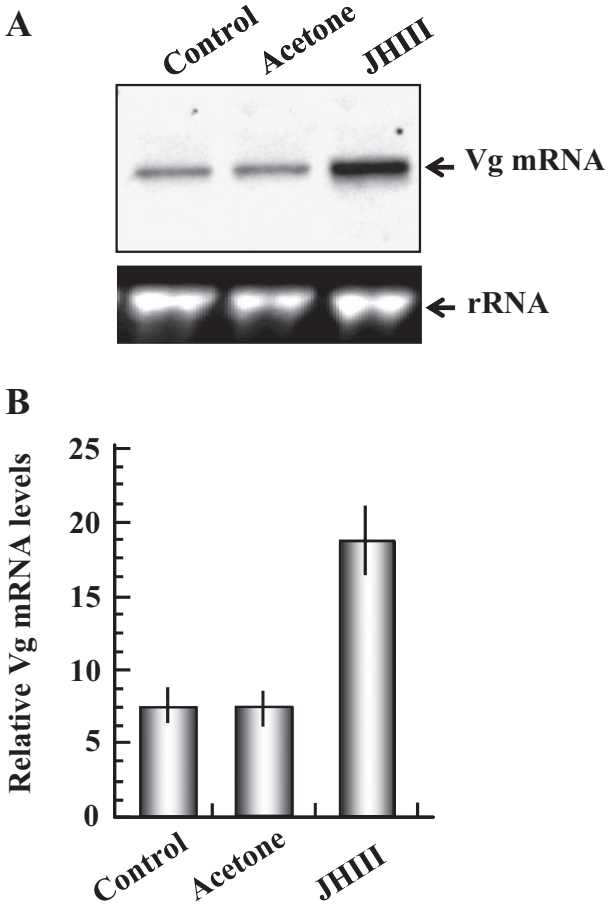


**Figure 6.** Effect of juvenile hormone III (JH III) on *Nilaparvata lugens vitellogenin* (*NlVg*) gene transcription. (A) Northern blot analysis shows the effect of JH III on *Vg* gene expression compared with those of untreated control and acetone-treated females. Ribosomal RNAs are shown as internal control after staining with ethidium bromide. (B) Quantification of NlVg mRNA levels from the Northern blots. The bars indicate the average values of three independent experiments ± SE.

5′-end sequence and the AP1 to achieve the complete 5′-end part. The amplification conditions used were 1 min at 94 °C, followed by 32 cycles of 94 °C for 30 s and 68 °C for 2 min. The amplified PCR products were cloned into the pT7Blue vector (Novagen, Madison, WI, USA) or pTA2 vector (Toyobo, Osaka, Japan) and sequenced. The overlapping sequences of the above three PCR fragments were assembled to obtain the full-length *NlVg* cDNA sequence.

### *Sequence analysis*

All sequences were analysed with the BCM search launcher computer program (http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html) and GENETYX v. 5.1 program (Genetyx Corporation, Tokyo, Japan). Sequences were checked for their homology with other Vgs using a FASTA or BLAST homology search on the DDBJ database (http://www.ddbj.nig.ac.jp/index-e.html). The signal peptide position was predicted using the SIGNALP computer program. Mass/isoelectric point was conducted using ExPASy-COMPUTE pI/Mw (http://www.expasy.ch/tools/pi_tool.html). The NCBI CDD search was used to identify the conserved regions in NlVg. The putative phosphorylation sites were detected by using the NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/).

### *Sequence comparison and phylogenetic relationship*

The Vg sequences used for comparison were from the following species (accession number in parentheses): *Periplaneta americana* (Vg1: AB034804; Vg2: AB047401), *Leucophaea maderae* (Vg1: AB052640; Vg2: AB194976), *Blattella germanica* (AJ005115), *Lethocerus deyrollei* (AB425334), *Graptopsaltria nigrofuscata* (AB026848), *Plautia stali* (Vg1: AB033498; Vg2: AB033499; Vg3: AB033500), *Riptortus clavatus* (U97277), *Homalodisca coagulat*a (DQ118408), *Nilaparvata lugens* (AB353856, the present study), *Anthonomus grandis* (M72980), *Tenebrio molitor* (AY714212), *Athalia rosae* (AB007850), *Pimpla nipponica* (AF026789), *Apis mellifera* (AJ517411), *Encarsia formosa* (AY553878), *Pteromalus puparum* (EF468683), *Solenopsis invicta* (Vg1: AF512520; Vg2: AY941795; Vg3: AY941796), *Aedes aegypti* (U02548), *Anopheles gambiae* (AF281078), *Anopheles albimanus* (AY691327), *Toxorynchites amboinensis* (AY691326), *Culex pipiens quiquefasciatus* (AY691324), *Bombyx mori* (D13160), *Lymantria dispar* (U90756), *Antheraea pernyi* (AB049631), *Antheraea yamamai* (AB055843), *Samia cynthia ricini* (AB055844), *Bombyx mandarina* (AB055845), *Saturnia japonica* (AB190809), *Actias selene* (EF523567), *Spodoptera litura* (EU095334), *Caenorhabditis elegans* (Vg1: AAB52675; Vg5: AAA83587; Vg6C AAQ91901), *Dermacentor variabilis* (Vg1: AAW78557; Vg2: ABW82681), *Rhipicephalus microplus* (ABS88989), *Tetranychus urticae*-Vg1-4 (AB505063-66), *Danio rerio* (NP_739573), *Gallus gallus* (AAA49139), *Ichthyomyzon unicuspis* (Q91062), *Macrobrachium rosenbergii* (BAB698831), *Pandalus hypsinotus* (BAD11098), *Cherax quadricarinatus* (AAG17936), *Metapenaeus ensis* (Vg1: AAM48287; Vg2 AAT01139; Vg3 (AAN40700), *Marsupenaeus japonicus* (BAD98732), *Litopenaeus vannamei* (AAP76571), *Fenneropenaeus merguiensis* (AAR88442) and *Penaeus mondon* (ABB89953). Sequences alignments were performed using the CLUSTALW program (Thompson *et al*., 1994). Phylogenetic analysis was performed using the neighbor-joining method by the Molecular Evolutionary Genetics Analysis (MEGA) v. 3.1 program (Kumar *et al*., 2001). For tree construction, Poisson correction distance, uniform rates amongst the sites, and a pairwise-deletion mode were used. The stability of the inferred phylogeny was estimated by the bootstrap test with 500 replications and a random seed value of 64238.

### *SDS-PAGE, immunoblotting and N-terminal sequence analysis*

Adult male and female whole bodies, as well as eggs (oocytes) were homogenized in the homogenization buffer (20 mM Tris/HCl pH 7.0, 2 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulphonyl fluoride), supplemented with a protease inhibitors cocktail (one tablet/50 ml of the buffer solution; Complete, Roche Diagnosis GmbH, Mannheim, Germany), using HG30 homogenizer (Hitachi Ltd., Tokyo, Japan). The homogenate was centrifuged at 10 000 *g* for 10 min. Protein contents in the supernatant were measured using the DC protein assay Kit (Bio-Rad, Hercules, CA, USA) according to the instruction of the supplier. Bovine serum albumin was used as a standard protein. The extracted proteins were mixed with equal volume of sample buffer [0.5 M Tris, pH 6.8, 10%SDS, 50% glycerol, 0.4 ml 2-mercaptoethanol, 0.05 % (w/v) bromophenol blue], denatured in boiling water for 10 min, then immediate cooled on ice for 2 min and stored at –20° C for future use. The haemolymph was sucked with a capillary from the adult females and was mixed in the above sample buffer at 1:50. The protein contents were measured as mentioned above. After boiling the haemolymph samples were also stored at –20 °C until required. A total of 10 μg for each sample was used for SDS-PAGE (15%) analysis. The gels were stained with Coomassie Brilliant Blue.

For immunoblot analysis, the separated male and female haemolymph and egg samples (10 μg per lane) were transferred on to the Immobilon-P membrane (Millipore, Billerica, MA, USA), and then incubated overnight at 4 °C with chicken anti-175 kDa NlVg primary antibody (diluted at 1:3000; a generous gift from Dr Roger F. Hou; see Cheng & Hou, 2005), followed by incubation for 1 h at room temperature with goat anti-chichen Immunoglobulin G horseradish peroxidase conjugated secondary antibody (Cappel, Westchester, PA, USA) (diluted at 1:50 000). The immune signals were detected using enhanced chemiluminescence plus detection reagents (Amersham-Pharmacia, Piscataway, NJ, USA) according to the supplier's protocol. To determine the N-terminal sequence of the 50 kDa Vg polypeptide, egg extracts (20 μg per lane) separated by SDS-PAGE were transferred to the polyvinylidene fluoride membrane (Millipore, Immobilon) and stained with Ponceau S (0.2% in 1% acetic acid). The bands corresponding to the 50 kDa Vg were cut out and applied to a gas phase amino acid sequencer (Perkin Elmer, Boston, MA, USA; 492 Procise).

### *Genomic digestion and Southern blotting*

The genomic DNA was isolated from the insect whole body with a Mammalian Genomic DNA Kit (Sigma, St. Louis, MO, USA). For Southern hybridization, 10 μg of genomic DNA was digested with restriction enzymes: *Eco*RV, *Not*I and *Xho*I (Toyobo). The digested DNA was precipitated with ethanol and separated on a 1% agarose gel. DNA was denatured and transferred to a Hybond $N^+$ membrane (Amersham, Little Chalfont, Buckinghamshire, UK) by capillary blotting. We used the Amersham Gene Images

AlkPhos Direct labeling and detection system (GE Healthcare, Buckinghamshire, UK). Briefly, the membrane was prehybridized in AlkPhos Direct hybridization buffer for 15 min at 60 °C, and hybridized overnight at 60 °C in the same buffer with a NlVg cDNA fragment (nt position: 1–1001) as a probe. After hybridization, the membrane was rinsed twice in the primary wash buffer for 10 min each at 60 °C and twice in the secondary wash buffer for 5 min each at room temperature. Then after incubation for 5 min in the detection reagent, the membrane was exposed to Hyperfilm-MP (Amersham) for 30 min.

*Total RNA preparation and Northern blot analysis*

Total RNA was isolated from the adult females and males, and from nymphs using isogen (Nippon Gene Co. Ltd, Toyama, Japan), and were stored at −80 °C until use. To determine the size, tissue-, sex- and stage-specific expression, and the developmental expression profile of NlVg mRNA, Northern blot hybridization analysis was performed. Aliquots of 20 μg of total RNA from adult females, males and nymphs were separated on 1% agarose/0.66 M formaldehyde gel in 3-morpholinopropane-sulfonic acid buffer. The separated RNA was transferred to Hybond $N^+$ membrane (Amersham-Pharmacia) through passive capillary transfer. The RNA blot was hybridized with a Vg cDNA probe (50 ng) synthesized from the 5′-end part of NlVg cDNA (nt position: 1–1078). For Northern analysis, we also used the Amersham Gene Images AlkPhos Direct labeling and detection system (GE Healthcare) as described above for Southern blotting.

*Transcriptional regulation of* NlVg *gene*

To study the hormonal regulation of NlVg gene transcription JH III (Sigma Chemical Co., St Louis, MO, USA) was applied topically to the abdominal sternites of 2-day-old adult females (brachypterous), a stage indicated to be sensitive for the JH analogue, methoprene, by Iwanaga & Tojo (1986). For treatment, JH III was dissolved in acetone to yield a final concentration of 200 ng/μl, and a droplet (0.5 μl) of JH III solution was applied after anaesthetizing with carbon dioxide. Control animals were treated with 0.5 μl acetone. The treated animals were kept separately under the same rearing conditions mentioned above. After 24 h of JH III application, total RNA was extracted from the treated animals and expression patterns of Vg mRNA were observed as described above.

## Acknowledgements

We thank Dr Y. Fukami, Mr S. Kawamoto and Dr T. A. Alexander, Research Center for Environmental Genomics, Kobe University, for amino acid analysis of polypeptides.

## References

Adams, T.S., Filipi, P.A. and Yi, S.X. (2002) Effect of age, diet, diapause and juvenile hormone on oogenesis and the amount of vitellogenin and vitellin in the twospotted stink bug, *Perillus bioculatus* (Heteroptera: Pentatomidae). *J Insect Physiol* **48**: 477–486.

Akasaka, M., Harada, Y. and Sawada, H. (2010) Vitellogenin C-terminal fragments participate in fertilization as egg-coat binding partners of sperm trypsin-like proteases in the ascidian Halocynthia roretzi. *Biochem Biophys Res Comm* **392**: 479–484.

Ayoade, O., Morooka, S. and Tojo, S. (1999) Enhancement of short wing formation and ovarian growth in the genetically defined macropterous strain of the brown planthopper, Nilaparvata lugens. *J Insect Physiol* **45**: 93–100.

Babin, P.J., Bogerd, J., Kooiman, F.P., Van Marrewijk, W.J.A. and Van der Horst, D.J. (1999) Apolipophorin II/I, apolipoprotein B, vitellogenin, and microsomal triglyceride transfer protein genes are derived from a common ancestor. *J Mol Evol* **49**: 150–160.

Barr, P.J. (1991) Mammalian subtilisins: the long-sought dibasic processing endoproteases. *Cell* **66**: 1–3.

Bertuso, A.G. and Tojo, S. (2002) The nature and titer of juvenile hormone in the brown planthopper, *Nilaparvata lugens* (Homoptera: Delphacidae) in relation to wing morphogenesis and oocyte development. *Appl Entomol Zool* **37**: 117–125.

Byrne, B.M., Gruber, M. and Ab, G. (1989) The evolution of egg yolk proteins. *Progr Biophys Mol Biol* **53**: 33–69.

Chen, J.-S. and Raikhel, A.S. (1996) Subunit cleavage of mosquito pro-vitellogenin by a subtilisin-like convertase. *Proc Natl Acad Sci USA* **93**: 6186–6190.

Chen, J.S., Sapington, T.W. and Raikhel, A.S. (1997) Extensive sequence conservation among insect, nematode and vertebrate vitellogenins reveals ancient common ancestry. *J Mol Evol* **44**: 440–451.

Cheng, D.J. and Hou, R.F. (2005) Determination and distribution of a female-specific protein in the brown planthopper, *Nilaparvata lugens* Stal (Homoptera: Delphicidae). *Tissue Cell* **37**: 37–45.

Cheng, S.N., Chen, J.C., Si, H., Yan, L.M., Chu, T.L., Wo, T.C. *et al*. (1979) Studies on the migration of brown planthopper, *Nilaparvata lugens* Stal. *Acta Entomol Sin* **22**: 1–21.

Denno, R.E., Olmstead, K.L. and McCloud, E.S. (1989) Reproductive cost of flight capability: a comparison of life history traits in wing dimorphic planthoppers. *Ecol Entomol* **14**: 31–44.

Denno, R.E., Roderick, G.K., Oimstead, K.L. and Dobel, H.G. (1991) Density-related migration in planthoppers (Homoptera: Delphacidae): the role of habitat persistence. *Am Nat* **138**: 1513–1541.

Dittmann, F., Trenczek, T. and Kleemann-Stumpf, I. (1985) Juvenile hormone-controlled vitellogenic cycles in *Dysdercus intermedius* (Heteroptera). *J Insect Physiol* **31**: 729–735.

Fairbairn, D.J. (1994) Wing dimorphism and migratory syndrome: correlated traits for dispersal tendency in wing dimorphic insects. In *Proceedings of Memorial and International Symposium on Dispersal Polymorphism in Insects, its Adaptation and Evolution* (Nakasuji, F. and Fujisaki, K., eds), pp. 143–152. Okayama University, Okayama.

Fujisaki, K. (1986) Reproductive properties of the oriental chinch bug, *cavelerius saccharivorus* Okajima (Heteroptera: Lygaeidae), in relation to its wing polymorphism. *Res Popul Ecol* **28**: 43–52.

Hagedorn, H.H., Maddison, D.R. and Tu, Z. (1998) The evolution of vitellogenins, cyclorrhaphan yolk proteins and related molecules. *Adv Insect Physiol* **27**: 335–384.

Harrison, R.G. (1980) Dispersal polymorphism in insects. *Ann Rev Ecol Syst* **11**: 95–118.

Hattori, M. and Sogawa, K. (2002) Oviposition behaviour of the rice brown planthopper, Nilaparvata lugens (Stal), and its electronic monitoring. *J Insect Behav* **15**: 283–293.

Hill, J.K. and Gatehouse, A.G. (1993) Phenotypic plasticity and geographical variation in the pre-reproductive period of *Autographa gamma* (Lepidoptera: Noctuidae) and its implications for migration in this species. *Ecol Entomol* **18**: 39–46.

Hirai, M., Watanabe, D., Kiyota, A. and Chinzei, Y. (1998) Nucleotide sequence of vitellogenin mRNA in the bean bug, *Riptortus clavatus*: analysis of processing in the fat body and ovary. *Insect Biochem Mol Biol* **28**: 537–547.

Ibanez, P., Garcera, M.D., Gavara, A. and Martınez, R. (1987) Effect of JH and PII on the protein content of haemolymph and ovaries of *Spilostethus pandurus*. *Comp Biochem Physiol* **88B**: 157–161.

Iwanaga, K. and Tojo, S. (1986) Effects of juvenile hormone and rearing density on wing dimorphism and oocyte development in the brown planthopper, *Nilaparvata lugens*. *J Insect Physiol* **32**: 585–590.

Johansson, A.S. (1954) Corpus allatum and egg production in starved milkweed bugs. *Nature* **174**: 174–189.

Johnson, C.G. (1969) *Migration and Dispersal of Insects by Flight*. Methuen and Co. Ltd., London.

Ketipearachchi, Y., Kaneda, C. and Nakamura, C. (1998) Adaptation of the brown planthopper (BPH), *Nilaparvata lugens* Stål (Homoptera: Delphacidae), to BPH resistant rice cultivars carrying *bph8* or *Bph9*. *Appl Entomol Zool* **33**: 497–505.

Kisimoto, R. (1965) Studies on the polymorphism and its role in the population growth of the brown planthopper, *Nilaparvata lugens* Stål. *Bull Shi Natl Agri Exp Stat* **13**: 1–107.

Kisimoto, R. (1976) Synoptic weather conditinso inducing long-distance immigration of planthoppers, *Sogatella furcifera* Horvath and *Nilaparvata lugens* Stal. *Ecol Entomol* **1**: 95–109.

Kumar, S., Koichiro, T., Ingrid, B.J. and Masatoshi, N. (2001) *MEGA2: Molecular Evolutionary Genetics Analysis Software*. Arizona State University, Arizona.

Kunkel, J.G. and Nordin, J.H. (1985) Yolk proteins. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, Vol. 1 (Kerkut, G.A. and Gilbert, L.I., eds), pp. 83–111. Pergamon press, New York, NY.

Lee, J.M., Nishimori, Y., Hatakeyama, M., Bae, T.-W. and Oishi, K. (2000) Vitellogenin of the cicada *Graptopsaltria nigrofuscata* (Homoptera): analysis of its primary structure. *Insect Biochem Mol Biol* **30**: 1–7.

Naeemullah, M., Sharma, P.N., Tufail, M., Mori, N., Matsumura, M., Takeda, M. *et al*. (2009) Characterization of brown planthopper strains based on their differential responses to introgressed resistance genes and on mitochondrial DNA polymorphism. *Appl Entomol Zool* **44**: 475–483.

Nagaba, Y., Tufail, M., Inui, H. and Takeda, M. (2010) Hormonal regulation and effects of 4 environmental polutants on vitellogenin gene transcription in the giant water bug, *Lethocerus deyrollei* (Heteroptera: Belostomatidae). *J Insect Conserv* (in press).

Ohkubo, N. (1967) Study on the density effect of the adult of the bown planthopper, *Nilaparvata lugens*. *Jap J Ecol* **17**: 230–233.

Raikhel, A.S. and Dhadialla, T.S. (1992) Accumulation of yolk proteins in insect oocytes. *Annu Rev Entomol* **37**: 217–251.

Roff, D.A. (1984) The cost of being able to fly: a study of wing polymorphism in two species of crickets. *Oecologia* **63**: 30–37.

Roff, D.A. (1986) Evolution of wing polymorphism and its impact on life cycle adaptation in insects. In *The Evolution of Insect Life Cycles* (Taylor, E. and Karban, R., eds), pp. 204–221. Springer, New York, NY.

Rouille, Y., Duguay, S.J., Lund, K., Furuta, M., Gong, Q. and Lipkind, G. (1995) Proteolytic processing mechanisms in the biosynthesis of neuroendocrine peptides: the subtilisin-like proprotein convartases. *Front Neur* **16**: 322–361.

Sappington, T.W. and Raikhel, A.S. (1998) Molecular characteristics of insect vitellogenins and vitellogenin receptors. *Insect Biochem Molec Biol* **28**: 277–300.

Sappington, T.W., Kokoza, V.A. and Raikhel, A.S. (1996) Molecular characterization of the mosquito vitellogenin receptor reveals unexpected high homology to the *Drosophila* yolk protein receptor. *Proc Natl Acad Sci USA* **93**: 8934–8939.

Sappington, T.W., Oishi, K. and Raikhel, A.S. (2002) Structural characteristics of insect vitellogenins. In *Progress in Vitellogenesis* (Raikhel, A.S. and Sappington, T.W., eds), Reproductive Biology of Invertebrates, Vol. XII. Part A, pp. 69–101. Science Publishers, Inc. Enfield, NH; Plymouth, UK.

Shinoda, T., Miura, K., Taylor, D. and Chinzei, Y. (1996) Vitellogenin and vitellin in the bean bug, *Riptortus clavatus* (Hemiptera: Alydidae): purification, immunological identification, and induction by juvenile hormone. *Arch Insect Biochem Physiol* **31**: 395–412.

Smolenaars, M.M.W., Madsen, O., Rodenburg, K.W. and Van der Horst, D.J. (2007) Molecular diversity and evolution of the large lipid transfer protein superfamily. *J Lipid Res* **48**: 489–502.

Snigirevskaya, E.S. and Raikhel, A.S. (2005) Receptor-mediated endocytosis of yolk proteins in insect oocytes. In *Progress in Vitellogenesis* (Raikhel, A.S. and Sappington, T.W., eds), Reproductive Biology of Invertebrates (Series Editors, Adiyodi KG, Adiyodi RG), Vol XII, Part B, pp. 199–228. Science Publishers, Inc. Enfield, NH; Plymouth, UK.

Sogawa, K. and Pathak, M.D. (1970) Mechanisms of brown planthopper (Hemiptera: Delphacidae) resistance in Mudgo variety of rice. *Appl Ent Zool* **53**: 145–158.

Sogawa, K. and Watanabe, T. (1992) Redistribution of rice planthoppers and its synoptic monitoring in East Asia. Technical Bulletin No. 131. Food & Fertilizer Technology Center, Taipei, Taiwan, pp. 1–9.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673–4680.

Tufail, M. and Takeda, M. (2002) Vitellogenin of the cockroach, *Leucophaea maderae*: nucleotide sequence, structure and analysis of processing in the fat body and oocytes. *Insect Biochem Mol Biol* **32**: 1469–1476.

Tufail, M. and Takeda, M. (2008) Molecular characteristics of insect vitellogenins. *J Insect Physiol* **54**: 1447–1458.

Tufail, M. and Takeda, M. (2009) Insect vitellogenin/ lipophorin receptors: molecular structures, role in oogenesis, and regulatiory mechanisms. *J Insect Physiol* **55**: 87–103.

Tufail, M., Raikhel, A.S. and Takeda, M. (2005) Biosynthesis and processing of insect vitellogenins. In *Progress in Vitellogen-*

*esis* (Raikhel, A.S. and Sappington, T.W., eds), Reproductive Biology of Invertebrates (Series Editors, Adiyodi KG, Adiyodi RG), Vol. XII, Part B. pp. 1–32. Science Publishers, Inc., Enfield; Plymouth.

Wada, T., Ito, K., Takahashi, A. and Tang, J. (2007) Variation of pre-ovipositional period in the brown planthopper, *Nilaparvata lugens*, collected in tropical, subtropical and temperate. *Asia J App Entomol* **131**: 698–703.

Wilson, K. and Gatehouse, A.G. (1993) Seasonal and geographical variation in the migratory potential of outbreak populations of the African armyworm moth, *Spodoptera exempta*. *J Anim Ecol* **62**: 169–181.

Zera, A.J. and Rankin, M.A. (1989) Wing dimorphism in *Gryllus rubens*: genetic basis of morph determination and fertility differences between morphs. *Oecologia* **80**: 249–255.

Zera, A.J. and Denno, R.F. (1997) Physiology and Ecology of dispersal polymorphism in insects. *Annu Rev Entomol* **42**: 207–230.

Zhao, X.C., Feng, H.Q., Wu, B., Wu, X.F., Liu, Z.F., Wu, K.M. *et al*. (2009) Does the onset of sexual maturation terminate the expression of migratory behaviour in moths? A study of the oriental armyworm, *Mythimna separata*. *J Insect Physiol* **55**: 1039–1043.