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Development of an ELISA for evaluating the reproductive status of female brown planthopper, *Nilaparvata lugens*, by measuring vitellogenin and vitellin levels

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Abstract

To evaluate the reproductive status of the female brown planthopper (BPH), *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), an indirect sandwich enzyme-linked immunosorbent assay (ELISA) for monitoring vitellogenin (Vg) and vitellin (Vt) was developed by using monoclonal antibodies and polyclonal antiserum made specifically against BPH Vt. The ovarian development of BPH was divided into five stages according to ovariole development and morphological characteristics. Stages I–III, IV, and V represented the pre-oviposition, peak oviposition, and post-oviposition stages, respectively. Levels of Vt in the ovary and Vg/Vt in the whole female body during the five ovary stages appeared to relate well with the corresponding ovarian stages, suggesting that ovarian development can be evaluated by measuring ovarian Vt or whole body Vg/Vt in BPH. With this ELISA protocol, the reproductive status of macropterous BPH captured in rice fields during immigration, dwelling, and emigration was determined based on the levels of Vg/Vt in individual females. The females were mainly in stages I and II, as was confirmed by ovarian dissection. Therefore, this study presented an alternative method for evaluating the reproductive status of BPH in rice fields, which is more precise, convenient, and efficient than conventional techniques, such as dissection and classification of ovaries.

Introduction

Vitellogenins (Vg), precursors of yolk proteins, have been shown to be very important for egg production in oviparous animals. In most insects, Vg is first synthesized in the female fat bodies, transported by the hemolymph to the ovary, and specifically sequestered by developing oocytes via receptor-mediated endocytosis and deposited as vitellin (Vt) (Sappington & Raikhel, 1998; Swevers et al., 2005). Insect Vt is structurally, biochemically, and immunologically very similar to corresponding Vg. The levels of Vg can serve as measures of reproductive fitness as they are a predictor of potential fecundity (Shapiro et al., 2000; Shapiro & Ferkovich, 2002). For Vg determination, three major techniques have been developed: radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), and Western blotting. ELISAs are widely used for the quantification of insect Vgs, such as those of *Aedes aegypti* (L.) (Ma et al., 1986), *Drosophila melanogaster* Meigen (Wu & Ma, 1986), and *Pteromalus puparum* (L.) (Dong et al., 2007).

The brown planthopper (BPH), *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), is a serious pest and disease vector in rice fields in Eastern Asia. It attacks rice plants by sucking fluid from the vascular bundle, and it

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transmits plant viruses. Brown planthoppers have both long-winged (macropterous) and short-winged (brachypterous) forms. Macropters are migratory and adapted to finding new habitats, whereas brachypters are sedentary and adapted to breeding in a suitable habitat (Mochida & Okada, 1979). Under the same conditions, brachypterous BPH females have higher fecundity and more rapid ovarian development than macropters (Bertuso & Tojo, 2002). There are many factors affecting the fecundity of BPH females, including temperature, food availability, the titer of juvenile hormone, and population density (Chen et al., 1979; Iwanaga & Tojo, 1986; Denno & Roderick, 1990; Ayoade et al., 1999). To estimate the population dynamics and fecundity potential of BPH females in rice fields, the reproductive status of macropters should be evaluated, especially during the migration season (Yin et al., 2008). However, there is no convenient method for evaluating the reproductive status of BPH females.

The insect ovary is regarded as a potential predictor to assess reproductive status (Büning, 1994). Its development is usually divided into 4–6 stages according to morphology and oogenesis (Dansereau & Lasko, 2005). However, the different stages lack clear criteria for classification, resulting in inaccurate definition of reproductive status. In this study, we developed an alternative method to evaluate the reproductive status of BPH in rice fields based on Vg/Vt levels. To this end, we prepared monoclonal antibodies against Vt and established a specific and sensitive sandwich ELISA for monitoring Vg/Vt in individual BPH females.

Materials and methods

Insects

The BPH populations were originally collected from rice fields in Hangzhou (120°10′E, 30°15′N), China, and maintained in an incubator at 26 ± 1 °C, 70–80% r.h., and L16:D8 on rice variety TN1 plants. To investigate the reproductive status of BPH in the rice field, macropters were sampled by light trapping on 5–25 June (immigration period), 10–20 August (dwelling period), and 1–15 September (emigration period) and collected by tapping rice seedlings with an aluminum tray (40 × 30 cm) on 15 August (dwelling) and 10 September (emigration) in 2009 in Fuyang, Hangzhou, China.

Sample preparation

Ovaries of BPH females were dissected in 0.02 M phosphate-buffered saline (PBS) at pH 7.4 and rinsed several times in the same PBS, as described by Dong et al. (2007). The ovaries or whole bodies of individual BPH females were homogenized in a glass tissue grinder in an ice bath. Homogenates were centrifuged at 10 000 ***g*** for 10 min at 4 °C, and the supernatants were stored separately at −70 °C for the Vg/Vt (total yolk protein, including hemolymph, fat body Vg, and ovarian Vt) determination.

Meanwhile, the ovaries of BPH females at different developmental stages were dissected in PBS and were photographed with a phase-contrast microscope (Leica, Nussloch, Germany) fitted with a light source. Digital images were captured using a digital camera and modified using Adobe Photoshop® (Adobe Systems, San Jose, CA, USA).

Purification of vitellin

The ovaries of the 3- to 6-day-old adult females were dissected in 0.1 M PBS, homogenized, and centrifuged at 10 000 ***g*** for 10 min at 4 °C. The homogenates were filtered through glass wool to remove lipids, diluted 20 times with ice-cold triple-distilled water, and kept overnight at 4 °C. The resulting precipitate was collected by centrifugation at 10 000 ***g*** at 4 °C and resolubilized in 0.1 M PBS. This high-salt extraction and low-salt precipitation were repeated three times. The precipitates from the last low-salt precipitation were resolubilized in 0.02 M PBS and stored at −70 °C for antibody preparation and as a reference standard for ELISA determination. The purified Vt concentration was determined with the Bio-Rad® protein assay (Bio-Rad Laboratories, Hercules, CA, USA) using BSA as the standard (Bradford, 1976).

Polyclonal and monoclonal antibodies against vitellin

The purified Vt (0.5 mg protein) emulsified in Freund's complete adjuvant (1 ml) was injected at different points on the back of approximately 6-month-old New Zealand male rabbits. The same antigen (1 mg) emulsified in Freund's incomplete adjuvant (1 ml) was injected on days 14 and 28. Blood was collected on days 42 and 49 through ear bleeding (30 ml). The antiserum was centrifuged at 2500 ***g*** for 10 min and stored at −70 °C. The affinity of antibodies was demonstrated by indirect ELISA (Xu, 1991). The antibody was made female specific by absorbing male homogenate solution as described by Wu & Ma (1986) and was purified by PROSEP-A spin columns (Millipore, Bedford, MA, USA).

Monoclonal antibodies against BPH Vg/Vt were prepared by the method of Dong et al. (2007). BALB/c mice were immunized subcutaneously with 50 μg of purified Vt emulsified in Freund's complete adjuvant and boosted at 2-week intervals with 50 μg of the same antigen preparation in Freund's incomplete adjuvant. Three days before fusion, mice were injected intraperitoneally with 50 μg antigen suspension in 100 μl sterile PBS, pH 7.4. Spleen cells from an immunized mouse were fused with mouse myeloma cell line SP2/0 in the presence of 50% PEG 3000.

Supernatants from hybridomas were screened for production of Vg/Vt-specific antibody using indirect ELISA and Western blotting.

SDS-PAGE and Western blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with 4% stacking gel and 8% separation gel was carried out according to Laemmli (1970). Samples were treated with an equal volume of the sample buffer (0.0625 M Tris–HCl, pH 6.8; 1% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.002% bromophenol blue) and boiled at 100 °C for 5 min. Gels were run in a solution of Tris–glycine buffer (0.025 M Tris, pH 8.3; 0.192 M glycine, 0.1% SDS) at 10 mA in stacking gel and at 20 mA in the separation gel until the dye front reached the bottom of the gel. Gels were stained with 0.1% Coomassie brilliant blue R250 in a solution of 50% ethanol and 10% acetic acid for 1 h and destained overnight with 10% ethanol and 10% acetic acid. Estimation of molecular weight was performed using the pre-stained marker and Quantity One® 1D Analysis software ver. 4.6 (Bio-Rad).

Proteins separated with SDS-PAGE were transferred onto a nitrocellulose membrane in Tris–glycine buffer (pH 8.3) for 20 min at 16 V in a semi-dry transfer blotter. The membrane was then blocked overnight with 5% nonfat dehydrated milk in PBS. The membrane was washed three times in PBS containing 0.05% Tween 20 (PBS-T) and incubated with anti-Vt monoclonal body raised against BPH for 1 h at room temperature. The membrane was washed three times with PBS-T and incubated for 2 h with the secondary antibody (horseradish peroxidase-conjugated anti-mouse IgG; Sigma, St. Louis, MO, USA) diluted 1:50 000 in PBS-T. The membrane was visualized with freshly prepared substrate (0.025% diaminobenzidine, 0.03% hydrogen peroxide, 0.025 M Tris–HCl). Distilled water was added to stop the reaction, and the membrane was dried with filter paper.

Indirect double-antibody sandwich ELISA

Polyvinyl 96-well microplates (NUNC, Roskilde, Denmark) were coated overnight at 4 °C with anti-Vt monoclonal antibodies developed against BPH as the primary antibody. Wells were washed three times with Tris-buffered saline containing 0.05% Tween-20 (TBST). Unoccupied protein binding sites were blocked by incubating with 1% BSA for 0.5 h at 37 °C. After washing with TBST, a dilution series of purified BPH Vt (from 0.78 to 200 $\mu g\ ml^{-1}$) was used as a reference standard. The samples were incubated in coated plates at 37 °C for 1.5 h followed by washing three times with TBST. The purified rabbit antiserum directed against the Vt of BPH (10 $\mu g\ ml^{-1}$) was then incubated in the wells followed by washing three times with TBST. Horseradish peroxidase-labeled goat anti-rabbit conjugate (Sigma) was added to the wells (100 µl per well) at 1:10 000 dilution for 1 h. After washing three times with TBST and another washing of TBS, the plate was developed using TMB Peroxidase EIA Substrate Kit (Pierce, Rockford, IL, USA) and incubated for 10 min at room temperature. The reaction was stopped with 2 M H_2SO_4, and the optical density was read at 450 nm on a Biotek® ELISA reader (Biotek, Winooski, VT, USA).

Statistical analysis

All data analyses were conducted using the DPS© package (version 8.01 for Windows) (http://www.china-dps.net) (Tang & Feng, 2007). The Vg/Vt levels of ovary and whole body at the five ovary developmental stages were analyzed with repeated measures one-way analysis of variance (ANOVA) and Duncan's multiple range test. To analyze the effects of ovary and whole body (factor A), developmental stages (factor B), and their interactions (A*B) on the levels of Vg/Vt, the data for each treatment were subjected to two-way ANOVA. The correlation between ovarian Vt levels and whole body Vg/Vt levels in various ovary developmental stages was analyzed using regression analysis. Differences in the levels of Vg/Vt between ovary and whole body extracts at the same developmental stage were compared by Student's t-test ($\alpha = 0.05$).

Results

Ovary development

The ovaries of brachypterous BPH can be divided into five stages according to ovariole development: (1) stage I, the ovary within 24 h after eclosion (Figure 1A), wherein only score I ovarioles existed (Figure 1F); (2) stage II, the ovary within 1–2 days after eclosion, wherein most ovarioles were in the stage of score II (Figure 1G), transparent, and indicative of little yolk protein deposition in the oocytes; (3) stage III, the ovary within 2–3 days after eclosion (Figure 1D), wherein most ovarioles were in the stage of score III (Figure 1H), containing one white and opaque oocyte, with nearly half of the oocytes filled with yolk protein and oocytes increasing gradually in size; (4) stage IV, the ovary within 3–10 days after eclosion, wherein most ovarioles contained one full-grown egg (Figure 1I), which was completely filled with yolk granules, the vitelline membrane being laid down around it; and (5) stage V, the ovary more than 10 days after eclosion, wherein most ovarioles were in the stage of score V (Figure 1J), where germaria had disappeared and only one matured oocyte existed.

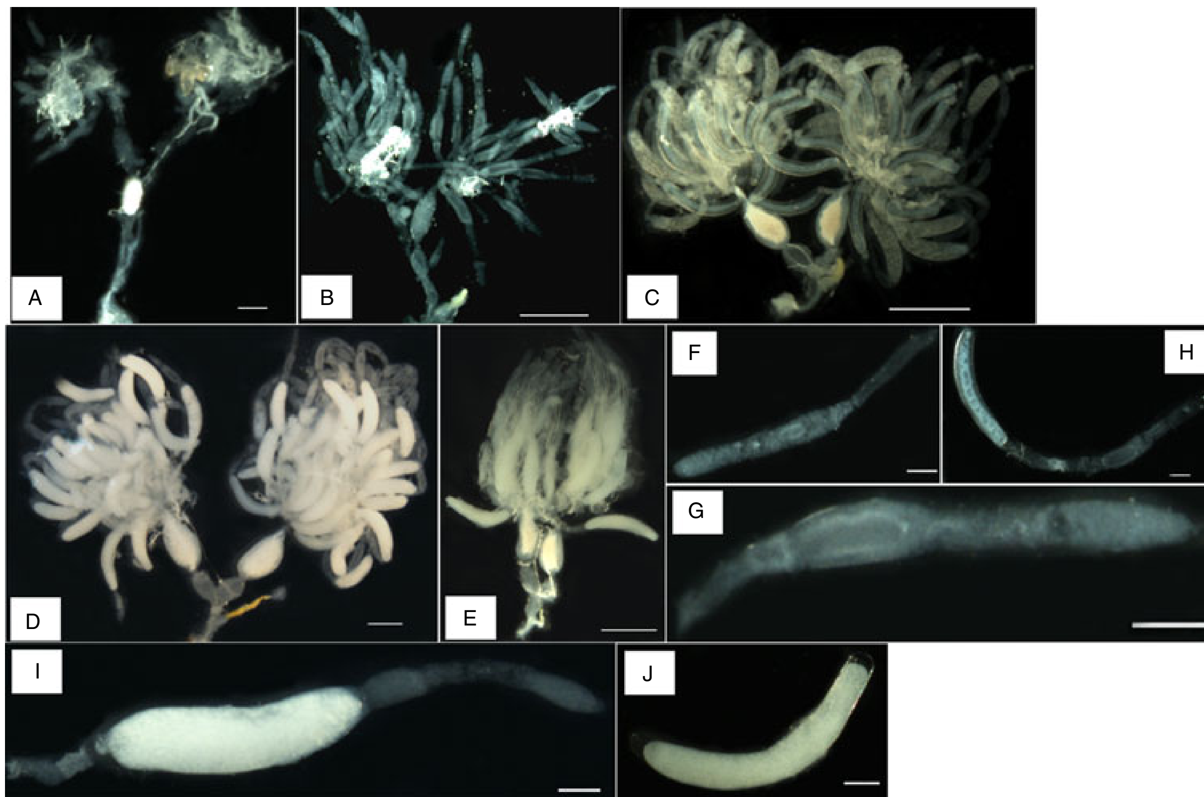

Figure 1 General morphology of ovaries and ovarioles in brachypterous *Nilaparvata lugens* at different developmental stages. (A) The ovary of freshly emerged brown planthopper, stage I; (B) 1–2 days after eclosion, stage II; (C) 2–3 days after eclosion, stage III; (D) 3–10 days after eclosion, stage IV; (E) 10 days after eclosion. (F–J) Representations of the developmental grades of egg chambers in stages I (F), II (G), III (H), IV (I), and V (J), respectively. Scale bars: 0.5 mm (A–E), 0.2 mm (F–J).

Characterization of monoclonal antibody against Vt

The purity of Vt, with one large and one small subunit, was shown on SDS-PAGE profiles (Figure 2B). By screening with indirect ELISA and Western blotting, a total of eight positive hybridoma cell lines were developed, which secreted antibodies specifically reacting to BPH's Vg/Vt based on a combination of gel electrophoresis and Western blotting (Figure 2A,C). Western blotting analysis also showed that the monoclonal antibodies only bind to the large subunit of Vg/Vt (Figure 2C).

Vg/Vt levels in ovary and whole body at five ovary developmental stages

The levels of ovarian Vt of macropterous BPH females at the five ovary developmental stages were detected with the indirect double-antibody sandwich ELISA (Figure 3). No Vt was detected in stage I ovaries, and the Vt titers per ovary from stage II-V were 5.1 ± 1.2, 25.4 ± 5.4, 47.4 ± 4.8, and 36.2 ± 3.1 µg, respectively. The ovarian Vt levels of the five developmental stages were significantly different ($F_{4,9} = 150.53$, $P<0.0001$). The titers of Vg/Vt extracted from the whole body of individual macropterous female at the various developmental stages were 7.6 ± 1.5 µg (stage II), 32.4 ± 5.8 µg (III), 64.6 ± 6.1 µg (IV), and 46.9 ± 3.1 (V), respectively (Figure 3). The levels of Vg/Vt among the five ovary developmental stages were also significantly different ($F_{4,9} = 170.66$, $P<0.0001$).

Both factor A (ovary and female whole body) and factor B (ovary developmental stages) affected yolk protein levels of individual BPH females, but their interaction had no significant effect (factor A: $F_{1,9} = 13.67$, $P = 0.021$; factor B: $F_{4,9} = 197.78$, $P = 0.0001$; A*B: $F_{4,9} = 1.62$, $P = 0.18$). In addition, a marked positive linear relationship existed between the ovarian Vt level and the whole body Vg/Vt level ($R = 0.997$, $F_{1,9} = 516.64$, $P = 0.0002$). It was suggested that ovary developmental stages were the main factors influencing yolk protein levels. Conversely, the ovary developmental stages can be evaluated by ovarian Vt levels or whole body Vg/Vt levels.

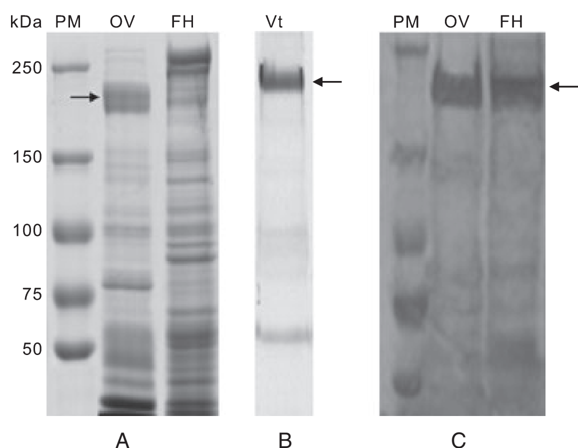

Figure 2 SDS-PAGE analysis with Coomassie brilliant blue staining of soluble proteins from stage IV ovaries, (A) female hemolymph and (B) purified vitellins, and (C) corresponding Western blot analysis with monoclonal antibodies against *Nilaparvata lugens* yolk soluble protein. PM: pre-stained high molecular weight standards; OV: soluble proteins of stage IV ovary extracts; FH: female hemolymph at 3 days after eclosion. Arrows indicate the large subunit of vitellin.

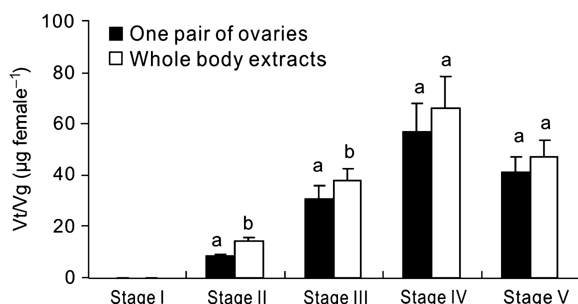

Figure 3 Mean (+ SEM) levels of vitellin in one pair of ovaries and vitellogenin/vitellin in whole body extracts of individual macropterous *Nilaparvata lugens* females at the five ovary developmental stages by indirect double-antibody sandwich ELISA. Means are based on 10 individual measurements. Means capped by different letters are significantly different (paired Student's t-tests: P<0.05).

Evaluation of reproductive status of field BPH females

According to the whole body Vg/Vt level of macropterous BPH females, most of the BPH collected from rice fields by light traps between 5 and 25 June were in stage II; no macropterous BPH were found in stage I, IV, or V (Figure 4). This may imply that most BPH were just immigrating from other areas. The macropterous BPH trapped during the dwelling period were in stages I and II, and only a few in stage III were detected. All five ovary developmental stages of macropterous BPH were collected directly from rice seedlings by tapping with an aluminum tray, and over half of them were in stage IV, indicating that they were at the peak of oviposition. During the BPH emigration season, about one-third of the macropterous BPH captured by light trap were in stage I, two-thirds of them were in stage II, and there were no other stages of macrophterous BPH. However, most of the macropterous BPH collected by tapping rice seedlings with an aluminum tray were in stage I, and only 20% of them were in stage II. This suggests that most of them were preparing to re-immigrate.

Discussion

Vitellogenin and Vt of BPH have been reported to consist of one large (about 175 kDa) and one small subunit (about 50 kDa), and they are expressed in a developmental and sex-specific manner (Cheng & Hou, 2005; Tufail et al., 2010). To determine the Vg/Vt level in BPH, polyclonal antisera against Vt were developed by Cheng & Hou (2005) and Dai & Yi (2006). However, the antisera exhibited low specificity, reacting with epitopes from other proteins, so it was difficult to quantitatively determine Vg/Vt levels in BPH females. In this study, we prepared monoclonal antibodies against BPH Vt, which showed a high sensitivity and accuracy, as they only reacted to the large subunit of Vg/Vt and showed no cross-reaction to other proteins in BPH females. With this monoclonal antibody as the basis, we developed an easy and precise ELISA protocol to assess the reproductive status of BPH females by measuring their levels of Vg/Vt. With this protocol, the reproductive status of macropterous BPH trapped in rice fields by light during the immigration, dwelling, and emigration periods was estimated, and the results were consistent with ovary dissection and classification by the method of Chen et al. (1979). This is a relatively quick technique for determining females' reproductive status, because in a single day, many individuals can be processed; ovary dissection and classification are not feasible for a large number of samples, and they are easily misjudged by investigators.

The reproductive status of BPH females has been the main factor used to determine natural population dynamics and ecological infrastructure of BPH females in rice fields (Chelliah et al., 1980), and reproductive status was also related to migration of BPH (Wada et al., 2009). Several indicators and methods have been adopted to evaluate the reproductive status of BPH females, such as ovary dissection and classification, and their pre-oviposition period (Chen et al., 1979; Wada et al., 2007). The BPH ovary consists of a number of ovarioles, and an ovariole is made up of a terminal filament, germarium, vitellarium, and ovariole pedicel (Mochida & Okada, 1979). The ovarian

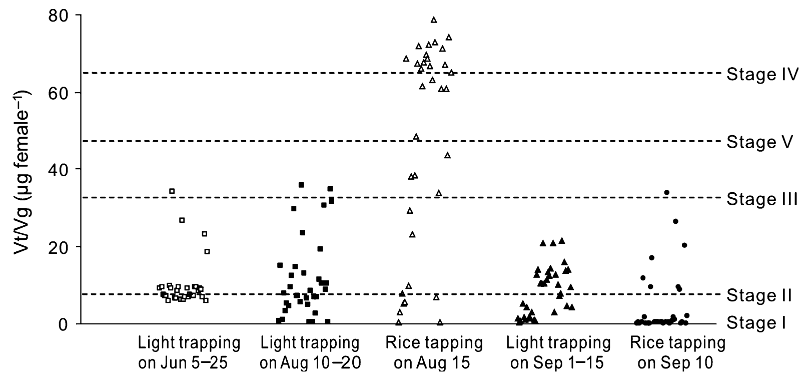

Figure 4 Levels of vitellogenin/vitellin for whole body extracts of field macropterous *Nilaparvata lugens* females collected with light trap or by tapping rice seedlings with an aluminum tray in 2009. Levels of Vg/Vt were monitored by direct ELISA using monoclonal antibodies against vitellin. Stages I-V correspond to the levels of Vg/Vt of individual brown planthoppers (Figure 3). Each point represents the levels of Vg/Vt from an individual brown planthopper, and more than 30 individuals were measured in each field sample.

development of BPH females was arbitrarily divided into four stages according to vitellogenesis in ovarioles (Iwanaga & Tojo, 1986), and five stages were identified on the basis of yolk protein content in oocytes (Chen et al., 1979). In this study, the ovarian development was also divided into five stages. Stages I-III, IV, and V represented the pre-oviposition, peak oviposition, and post-oviposition stage, respectively. Ovarian development has been used to evaluate the effect of juvenile hormone on the fecundity of BPH females to forecast the reproductive status and outbreak of BPH (Iwanaga & Tojo, 1986; Ayoade et al., 1999; Pradeep & Nair, 2005). Our study showed that the level of ovary Vt and female whole body Vg/Vt at five ovary developmental stages can be aligned well with the corresponding ovary stages. It was suggested that ovary development can be evaluated by ovarian Vt or whole body Vg/Vt in BPH. However, for determining ovarian Vt, it took more time to dissect the ovary. Therefore, whole body Vg/Vt was more convenient to evaluate the reproductive status of BPH.

The use of ELISA to assess the level of yolk protein as a measure of female reproductive status and as a predictor of potential fecundity has also been reported in other insects. In pharaoh ant queens (*Monomorium pharaonis* L.), ELISA has been used to assess reproductive response in relation to the presence or absence of workers (Jensen & Børgesen, 1995). In the spined soldier bug, *Podisus maculiventris* (Say), an ELISA was used to quantify the reproductive response of females to diets of differing quality (Shapiro et al., 2000). In *Orius insidiosus* (Say), anti-Vt monoclonal antibodies and quantitative ELISA were developed to assess the reproductive response to dietary and other rearing conditions and to assist in quality control and diet development for mass rearing (Shapiro & Ferkovich, 2002).

The pre-oviposition period (the time between adult emergence and the laying of fully developed eggs) averages 3–4 days for brachypterous females, but 3–10 days for macropters at constant temperatures of 20–33 °C (Mochida & Okada, 1979). For migratory macropters, the pre-oviposition period was difficult to estimate because it can be prolonged to 1 month or longer when there is no appropriate habitat available (Kisimoto & Sogawa, 1995). In addition, macropterous BPH originating from temperate and subtropical East Asia had a longer pre-oviposition period than those of tropical populations (Wada et al., 2007). The pre-oviposition period of BPH was influenced by food quality and probably by nymphal rearing densities and resistant rice varieties (Lu et al., 2004; Wada et al., 2007). In this study, we evaluated whether BPH was in the pre-oviposition period in rice fields through determining the levels of Vg/Vt. The pre-oviposition period was divided into three stages according to the level of whole body yolk protein level. When the levels of whole body Vg/Vt were in stage I or II, macropterous BPH needed at least 3 days to lay eggs. However, when levels of whole body Vg/Vt were in stage III, macropters needed only 1 day to lay eggs. Therefore, with this ELISA protocol, it is easy to evaluate the time needed for female BPH to reach reproductive maturity, and it is more accurate than the assessment of pre-oviposition period by conventional methods.

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