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# Simplified morphological criteria for ovarian classification in evaluating reproductive age groups of brown planthopper, *Nilaparvata lugens* (Stål)

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**ABSTRACT**: Understanding the morphological changes in the female reproductive system is useful for predicting changes in population levels of the brown planthopper, *Nilarparvata lugens*. This study used morphological criteria to develop a simplified classification of ovarian development using only three phases based on age groups. In addition, the time required to complete each phase was investigated. Changes in the oviposition activity were examined in the female reproductive organs of virgin and mated macropterous *N. lugens*. Prior to dissecting *N. lugens*, side views of the females were captured, and the maximum abdominal width and contact angle (dragged from the center of annal tube to either side of body wall of the insect abdomen) were measured. Out of 1,032 samples, the ovaries of 300 virgin females did not develop to maturity, while 732 mated ones were categorized into three phases: I (pre-ovulation), II (ovulation and oviposition), and III (post-oviposition). The maximum time taken to develop during phase I was 5 days. Phase II period was prolonged for up to 15 days with an average of 6.9 days. The number of eggs laid per female varied from 3 to 208 with an average of 96±4.9 eggs. Females entered phase III within 5–17 days. The maximum abdominal width and contact angle of *N. lugens* were significantly different across three ovarian phases. These criteria provide a basis for identifying the ovarian phase and predicting the population dynamics of *N. lugens* as a key determinant in decision-making.

**KEYWORDS**: brown planthopper, *Nilaparvata lugens*, morphological criteria, ovarian classification, reproductive age group

## INTRODUCTION

The physiological age of animals is a key determinant in providing useful information on fertility and predicting insect pest population sizes [1]. As with all organs, changes in ovarian morphology can be used to determine the physiological age of insects which reflect the physical and genetic requirements during oocyte growth and the timing of ovulation [2–4]. The brown planthopper, *Nilaparvata lugens* (Stål) is considered a major threat to rice production throughout Asia, causing economic damage to rice crops directly by sap-feeding and transmitting plant-pathogenic viruses [5, 6]. The adult of *N. lugens* has 2 wing forms that are considered an adaptive trade-off between migration and reproduction [7, 8]. The female reproductive system of *N. lugens* is termed a telotrophic type [9–11]. There is a pair of ovaries, and each ovary is made up of many series of egg tubes or ovarioles. The number of ovarioles per female ranges from 43 to 54 and 46 to 54 in the macropterous and brachypterous forms, respectively [12, 13]. Even though there are some differences in female fertility between macropterous and brachypterous *N. lugens* [14], they share the same pattern of ovarian development. Each ovariole is composed of a terminal filament, germarium, vitellarium, and pedicel. Oogenesis in *N. lugens* starts with oogonia, located in the most anterior region of the germarium; they transform into oocytes and move to the vitellarium region, which carries a long string of oocytes with each oocyte developing into a mature oocyte [15].

Based on the pattern of ovarian development, a method of dissection is used to determine the physiological age-grading of insect ovaries [16]. Iwanaga and Tojo [17] observed the accumulation of yolk protein and coded the stages of ovarian development for *N. lugens* based on the degree of vitellogenesis with a score from 0 to 3. However, the determination of the physiological age of female insects is of both theoretical and practical interest. Thus, a basic requirement of ecological studies on insect populations with overlapping generations is that adult insects collected in the field must be referable to the known age structure in a population [18]. To date, only the criteria developed by Chen and coworkers in 1979 [19] have been used to determine the physiological age of *N. lugens* ovaries, and this information has been applied there-

after for forecasting migration by categorizing into 3 field population types: immigration; part-emigration, part-sedentary, and local breeding; and emigration [20–22]. Though data on the age structure of an insect population are useful for insect pest management, until now, there is still a lack of alternative criteria that can be used to identify the population age and to evaluate the threats of this insect pest species to rice crops. According to Chen et al [19], the ovarian development of *N. lugens* was classified into 5 grades with thorough details. However, it requires enough technicians to monitor ovarian activity frequently, ideally daily. Consequently, there is a need for the development of a variety of different criteria that could categorize a broad age group and predict how population dynamics will change since this pest has emerged in field crops. Against this background, the first aim was to classify the phase of ovarian development of *N. lugens* into simplified morphological criteria based on 2 aspects. First, these criteria can be used to determine the age structure of the field populations and to indicate what may be expected in the future. In general, reproduction is a very broad term that can apply to any behavioral and physiological changes that occur in insect females before, during, and after egg-laying [23]. Second, a recognition technique is necessary to make it possible for any investigator to easily notice differences among the ovarian phases. To develop a more precise, reliable criterion, the second key aim of the current research was to determine the period to complete each phase. The simplified morphological criteria used in this research enable us to predict the occurrence of offspring of *N. lugens* and provide key information for developing an early warning system for *N. lugens*.

## MATERIALS AND METHODS

### Field surveys and insect cultures

Adults of *N. lugens* were collected from rice fields in the Central Plains of Thailand during 2017–2019 and cultured on rice seedlings (aged 4–5 weeks) of *Oryza sativa* L. c.v. TN1 under laboratory conditions (20–35 °C air temperature and 50–60% relative humidity).

### Classifying the ovarian development phase to establish new morphological criteria

Newly emerged macropterous female adults were divided into 2 groups. The virgin female adults in group 1 were reared individually ($n = 300$), while a total of 723 newly emerged female adults in group 2 were mated in pairs in a rearing tube with virgin males (aged 2–5 days). Each tube contained rice seedlings. Then, during 18 consecutive days, new rice seedlings were replaced daily, and the egg-laying activity of *N. lugens* on the old rice plants was checked daily under a stereo microscope to trace and confirm ovarian developmental phases. Daily, females were randomly selected and euthanized by placing them in a freezer at 0 °C (1 h) before preserving in 75% ethyl alcohol for 5–7 days. Of all females, digital photographs (side view) were taken under a stereo microscope (SZX16) with a digital camera DP22 (Olympus, Japan), and the maximum abdominal width and contact angle (from the center of the anal tube to either side of the body wall of the insect abdomen) were measured (Fig. 1a). Thereafter, all preserved specimens were dissected to investigate the morphological changes of the reproductive organs for each 24 h interval for 18 consecutive days (Fig. 1b).

For dissection of the organs in the reproductive system of *N. lugens* (Fig. 2a–c), each adult female was transferred to the dissecting plate and dropped with 75% ethyl alcohol. Then, the abdomen was opened using a dissecting pin to remove the integument. Thereafter, one dissecting pin was used to gently pull the intact reproductive organs away from other internal structures. After that, the calyx and lateral oviducts flipped down to make a Y-shaped tubular structure to separate into the left and right ovaries. A drop of 75% ethyl alcohol was used during dissection to prevent the drying out of the internal reproductive organ. Digital photographs of all dissected organs in each female were taken using the stereo microscope and digital camera. The ovarian development was graded based on differences in morphological structure corresponding to egg-laying behavior into 3 phases: phase I (pre-ovulation), phase II (ovulation and oviposition), and phase III (post-oviposition). The structure of the internal reproductive organs in *N. lugens* females is shown in Fig. 2d.

### The measurement period for completing each ovarian developmental phase

To contribute to the forecasting work, we used individual records of macropterous females from the previous section to analyze the duration of each ovarian phase. All essential reproductive activities (egg formation, ovulation, and oviposition) must occur in the 2 insect groups: virgin females and mated females, respectively. The ability of *N. lugens* to lay a series of eggs was also investigated by replacing new rice plants in the mating tube and counting the number of eggs deposited on the old rice plants every 24 h during the oviposition period. The total number of eggs laid per female throughout the entire oviposition period was assessed only from dissected females with ovarian development in phase III.

### Comparison of two morphological criteria for ovarian classification in *N. lugens*

The ovarian classification systems reported by Chen et al [19] were compared with those of the current study to understand the concept and conditions for application in field research.

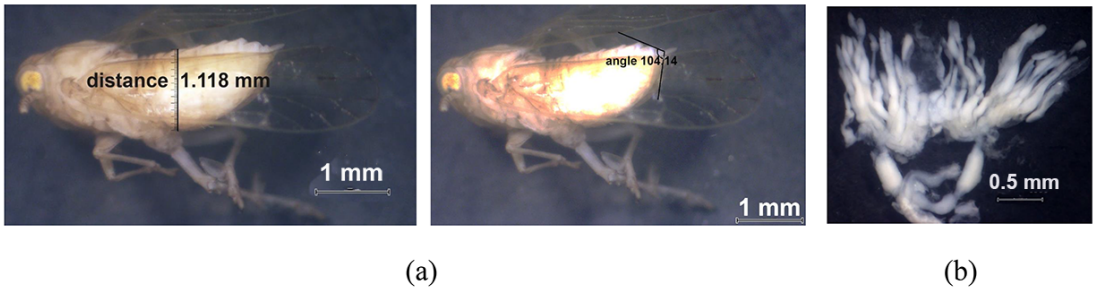


**Fig. 1** External morphology of *N. lugens* (BPH). (a) Side-view of the abdomen. Maximum abdominal width and contact angle are measured because the side-view of the abdomen is difficult to measure in practice due to the convex shape. (b) Photograph of internal reproductive organs used for later categorization of ovarian phase.

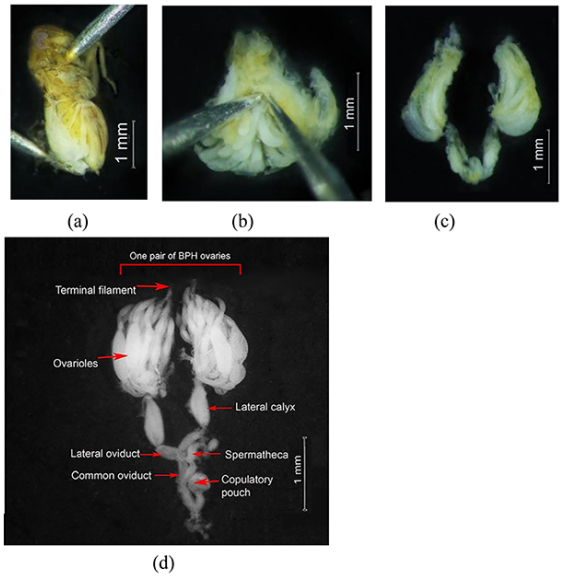


**Fig. 2** Dissection performed to determine the physiological age of *N. lugens* ovaries. (a) Removal of wings and exoskeletal plates covering the abdomen. (b) Calyx and lateral oviduct connecting ovaries as inverted Y-shape. (c) Flipped ducts in Y-shaped tubular structure separated into left and right ovaries. (d) Internal reproductive system of *N. lugens* adult female, which consists of a pair of ovaries connected to the anterior part of the lateral oviduct (calyx).

### Statistical analysis

One-way ANOVA was used to test for significant differences in the maximum abdominal width and contact angle at a test level of $p < 0.05$. The relationship between the oviposition period and the total number of eggs laid per female was determined using Pearson correlation analysis. The correlation level for significance was set to $p < 0.05$. All analyses were performed in SPSS 17.0 for Windows.

## RESULTS

### Novel simplified morphological criterion for classification of ovarian phases in *N. lugens*

The experiments indicated that the ovaries of 300 virgin females did not develop to maturity (Fig. 3). Thus, the criteria for ovarian development were obtained from the 732 mated *N. lugens* in group 2. As changes in the morphological structure of the female reproductive organs corresponded with ovipositional history, the simplified morphological criteria were divided into the following 3 phases.

#### *Phase I pre-ovulation*

Phase I was characterized by a degree of oocyte development that caused a series of structural changes in the ovaries. At the beginning of this phase, the newly emerged adult females had a low level of ovarian development with whitish coloration (Fig. 4a). Then, a long string of oocytes continued to increase in size in the vitellarium (Fig. 4b). Toward the end of phase I, the oocytes developed into mature eggs, and these mature oocytes prepared to enter the oviduct (Fig. 4c). The distinct morphological features during development in phase I included the presence of developing oocytes with few mature oocytes and the shape of the calyx and epithelial plug.

#### *Phase II ovulation and oviposition*

Two important processes took place in this phase (ovulation and subsequent oviposition). Simultaneously, the production of new oocytes continuously took place and developed into a mature egg progression as a long string in the ovariole to replace the ovulated egg. Therefore, ovulating *N. lugens* females produced high numbers of mature oocytes and deposited high numbers of eggs during this phase. The distinct morphological features of the ovary in phase II were the presence of high numbers of mature oocytes and the elongation of calyx (Fig. 5a and b).

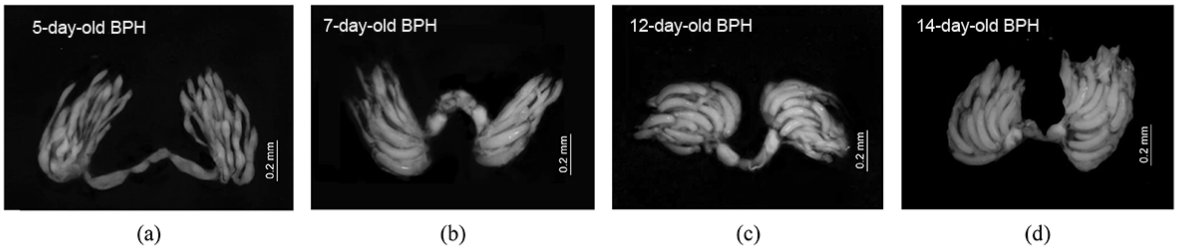


**Fig. 3** Ovaries of virgin adult *N. lugens* females. (a–d) Samples of *N. lugens* at 5, 7, 12, and 14 days, respectively, after emergence that were unable to produce mature oocytes.

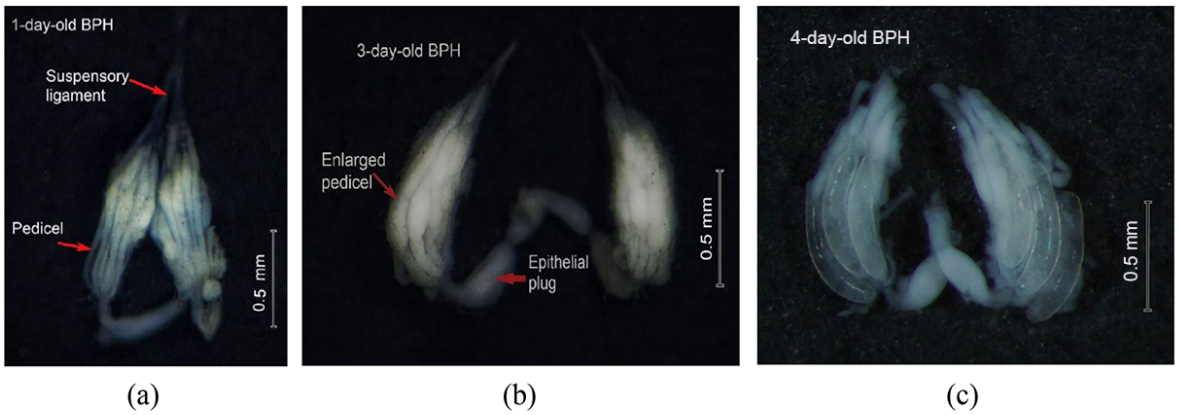


**Fig. 4** Morphology of internal reproductive organs of an *N. lugens* female in phase I before egg laying. (a) Oocyte development of adult female aged 1 day showing little progress as indicated by a thin pedicel. (b) Ovaries filled with developing oocytes at different stages. (c) Fully developed ovaries containing some full-grown, banana-shaped eggs.

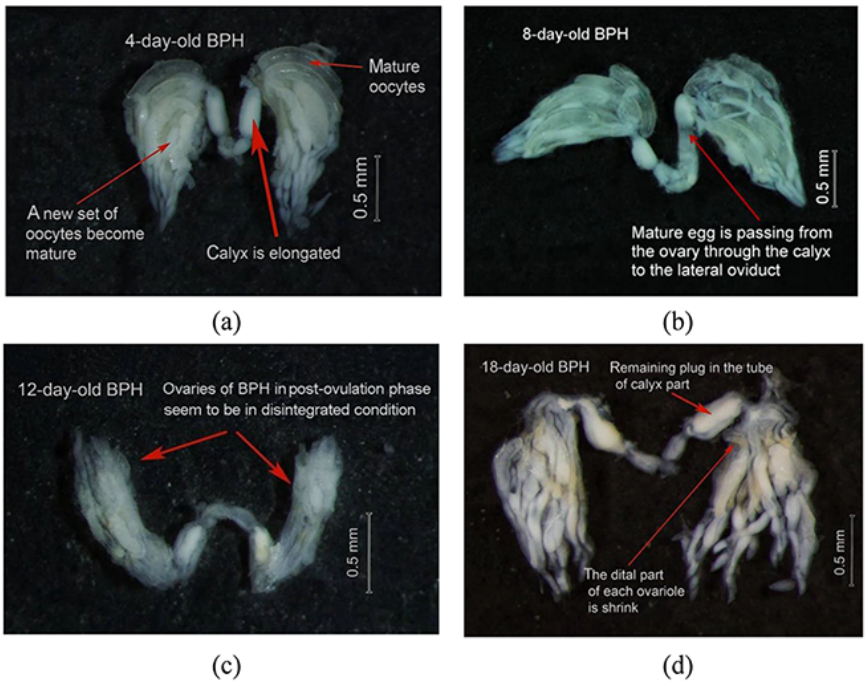


**Fig. 5** Morphology of female internal reproductive organs of *N. lugens*. (a and b) Ovary in phase II mature eggs rupturing the epithelial plugs and expelled into the oviduct. (c and d) Ovary in phase III calyx elongated and filled with yellowish-white epithelial plug.

***Phase III post-oviposition***

Phase III was the terminal phase of oviposition. The ovaries of the females after completion of oviposition are shown in Fig. 5c and d. Changes in the morphology of the ovarioles and calyx were used as indicators of past reproductive activities. The ovaries are in disintegrated condition which is a sign of no further role in ovulation and oviposition.

In addition to categorizing the ovarian phase based on the changes in the internal reproductive organs, the results also showed that changes in the external morphology of the abdomen could be related to the development phase of the ovary. Table 1 summarizes the maximum abdominal width and contact angle of the females with ovarian development in each phase. There were highly significant differences among the ovarian development phases in terms of the maximum abdominal width ($F_{2,371} = 97.131$, $p < 0.0001$) and contact angle ($F_{2,371} = 98.498$, $p < 0.0001$).

**Period to develop each ovarian development phase**

Out of a total of 732 *N. lugens* females, 175, 379, and 178 were found to have ovaries in phase I, II, and III, respectively. In the current study, most *N. lugens* females died with mature eggs likely due to individual variations in favorable physical conditions. As a result, the highest numbers of females described in our findings were in phase II. The time ranges required for adults to reach specific ovarian development phases are shown in Fig. 6a. In phase I, the maximum time taken by females to develop in the pre-ovulation phase was 5 days with the majority falling within the range 1–3 days after adult emergence. The longest duration for ovarian development occurred during phase II, lasting approximately 15 days after the pairing occurred. The results revealed that 3.17% of the females began depositing eggs on the second day after mating. During the terminal phase of oviposition, females entered the post-oviposition phase between 5 and 17 days after emerging as adults.

In total, 16,925 eggs were produced by the 178 *N. lugens* females with ovarian development in phase III. The total number of eggs deposited in consecutive oviposition by individual females of *N. lugens* varied from 3 to 208 with a mean (±SD) of 96.16±4.919 eggs laid per female. The egg-laying period for each female averaged 6.9 days, ranging from 1 to 13 days. Based on the Pearson correlation analysis, there was a strong positive correlation between the oviposition period and the total number of eggs laid by individual *N. lugens* ($r = 0.888$, $p < 0.0001$; Fig. 6b).

**Comparison of two morphological criteria for ovarian classification in *N. lugens***

Table 2 presents information on the staging classification of ovarian development in *N. lugens* and the application of 2 morphological criteria. The present study classified ovarian development into 3 phases, while Chen et al [19] identified it as 5 stages. However, both criteria have focused on physiological and morphological aspects that deserve greater consideration in the ovarian classification and categorization of the field population in *N. lugens*. The relationship between the developmental stages of the ovaries and migration status is drawn up by the criterion from Chen et al, aiming to predict population trends. In comparison, the criteria in the present study are designed to determine the field population from the phase of ovarian development with the reproductive age group. These criteria will be discussed further in the next section.

## DISCUSSION

A limited amount of simple and feasible criteria for ovarian classification which can be used to divide field populations into broad age groups has been a major constraint on gaining the necessary ecological data to make predictions before the outbreak of *N. lugens*. To develop an alternative criterion for ovarian classification, it needs to have a thorough understanding of the fundamental concept of reproductive biology. Our experiments supported data that mating plays an important role in stimulating reproductive physiological responses and releasing oviposition behavior in *N. lugens*. There are a number of reasons to explain why changes in oocyte development to maturity and egg-laying are acquired resources from male ejaculates. To begin with, the male accessory glands of *N. lugens* are responsible for synthesizing and secreting seminal fluid components and transferring them to females, serving a variety of functions associated with egg deposition [24]. The uptake of vitellogenin by developing oocytes also appears to be regulated by hormones, including juvenile hormone [25] and neurosecretory hormones [26] which are stimulated by a male accessory gland factor acquired during mating. This study establishes criteria for the future practical application in interpreting ecological field data by dividing it into 3 phases.

During the pre-ovulation phase, 2 indicators are considered including a linear series of developed oocytes with the presence of mature oocytes at the end of this phase and the presence of epithelial plugs in the calyx. These plugs are masses of inter-follicular that separate the mature oocyte from the pedicel and partly into the calyx [27]. The calyx is a sac-like structure in which the fully grown eggs of the ovarioles pass during ovulation [28]. Therefore, in phase I, the short and small size of the calyx is an indicator that the *N. lugens* female has not ovulated yet. Whilst phase II is denoted as the ovulation and oviposition phase. In our observations, the duct is stretched to about twice its normal length. In general, the generative follicular cells accumulate in the calyx and undergo autolysis after each ovulation [29]. At this stage, the cellular

**Table 1** Maximum abdominal width and contact angle of mated *N. lugens* in different ovarian development phases.

| No. | Age (days) | Phase | Maximum abdominal width (mm) | | Contact angle (°) | |
|---|---|---|---|---|---|---|
| | | | min–max | mean ± SD | min–max | mean ± SD |
| 175 | 1–5 | I | 0.361–1.67 | $0.741 \pm 0.18^{c}$ | 23.57°–115.67° | $53.56 \pm 18.18^{c}$ |
| 379 | 2–16 | II | 0.547–1.90 | $1.010 \pm 0.22^{a}$ | 40.78°–130.27° | $74.03 \pm 14.69^{a}$ |
| 178 | 5–18 | IIII | 0.440–1.95 | $0.966 \pm 0.22^{b}$ | 29.02°–132.07° | $70.79 \pm 17.09^{b}$ |

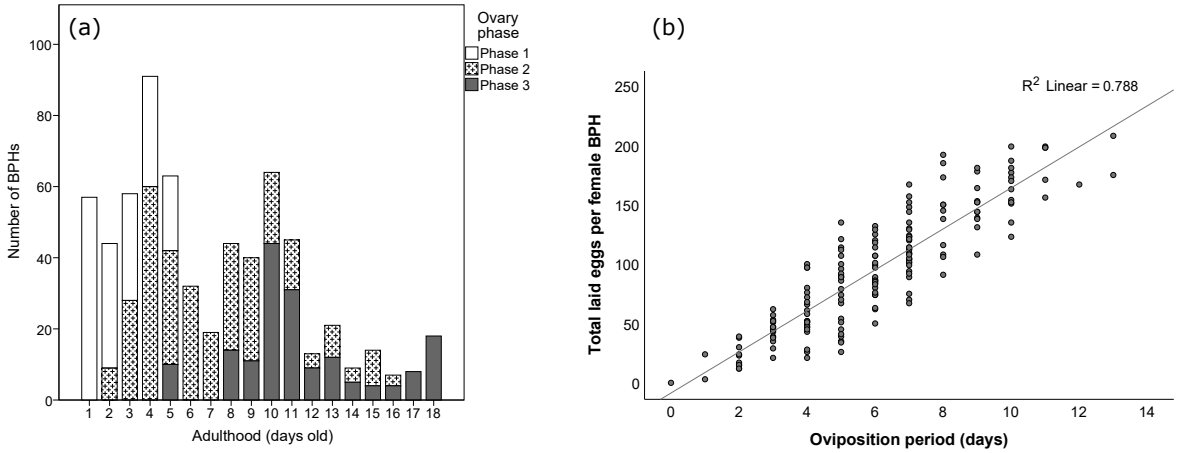


**Fig. 6** The estimated activity duration required to complete each phase of ovarian development. (a) The range of time required for ovarian development in phase 1 (white bar), phase 2 (bar with plus sign), and phase 3 (grey bar). Time is expressed as the number of days from the beginning of pairing in *N. lugens*. (b) The relationship between the oviposition period and the total number of laid eggs by individual *N. lugens* investigated from 178 females. Pearson correlation coefficient is 0.888 ($p < 0.0001$).

mass is bright yellow and reabsorbed through the ovariole epithelium during ovulation [27]. Therefore, during phase III, the epithelial plugs become yellowish and elongate in the similar shape of the stretched calyx. Besides, the pedicel, a simple tube at the terminal part of the ovariolar tube where the mature oocytes are lodged before passing into the calyx [30], is one of the good markers as it becomes withered after ovulation ends. The important data obtained from measuring abdominal circumstances would enable a rough distinction between ovarian development phase I and phase II in *N. lugens*. However, it is difficult to measure all *N. lugens* samples due to time-consuming. Another consideration is that the feeding and overall fitness of *N. lugens* are influenced by various factors such as rice varieties, population abundance, and the intracellular yeast-like symbiotes (YLS) in the fat bodies [31]. These factors could affect the body size of *N. lugens*. Therefore, the dissection technique remains the most precise approach.

To apply these current criteria for forecasting work, our second experiment revealed that the pre-ovulation period ranged from 2 to 5 days. The first clutch of eggs can typically be found within 2 days with

**Table 2** Points of similarity and difference between the present criterion and another existing criterion developed by Chen et al [19] for classifying ovarian development.

| | This study | Chen et al [19] |
|---|---|---|
| Indicator of criterion | Morphological change of reproductive organ relating to ovipositional history | Morphological change of reproductive organ |
| Classification | phase I to III | stage I to V |
| Guideline for applying in ecological study | 1) pre-ovulation<br>2) ovulation and oviposition<br>3) post-oviposition | 1) immigrant population<br>2) part-emigrant, part-sedentary and local breeding population<br>3) emigrant population |
| Appropriate implementation | both fresh and preserved specimen | fresh specimen |

an average oviposition period of 6.9 days. In comparison with other studies, Manjunath [32] reported that the pre-oviposition and oviposition periods of the macropterous *N. lugens* average 2.9 and 8.4 days, respectively. The time taken to complete the pre-oviposition and oviposition periods differs slightly between Manjunath's [32] and our study. This is because laboratory conditions vary in terms of temperature and humidity. Our results also agree with the observation of Mochida and Okada [15] in that the oviposition period has a significant positive correlation with the total number of eggs laid by individual *N. lugens*. Egg-laying by *N. lugens* in this study is consistent with the study by Lee and Park [33] in that *N. lugens* females could produce 150 to 250 eggs at 25 °C under laboratory conditions. Whilst Mochida and Okada [15] reported that *N. lugens* lay eggs throughout their lifespan ranging from 0 to 1,474.

Our results also suggest that the time required for ovarian development in each phase varies greatly among individuals of *N. lugens* females. A possible physiological explanation for these findings is that ovarian development relates to the different levels of juvenile hormones in the late nymphal instars, which play a role in regulating vitellogenesis [17]. Likewise, differences in the abdominal fat body of *N. lugens* may affect fertility as it is the exclusive site of vitellogenin synthesis [34]. The ability to intake phloem sap ingestion from the rice plant also varies among *N. lugens* which can affect their reproductive development [35]. This is due to the close association of *N. lugens* with YLS, which are found in the fat body cells. YLS are reported to play several important roles in *N. lugens* such as adapting to resistant rice varieties and contributing to the synthesis of essential amino acids that are vital for normal development [31]. Therefore, various activities such as feeding, egg development, and oviposition are influenced by the quantity of YLS in *N. lugens*. Moreover, population density and wing form of *N. lugens* are key factors that influence ovarian development. In field populations, *N. lugens* females appear to be sensitive to an increase in nymphal density, which lead them to develop long wings [36]. It has been shown that the pre-oviposition period in macropterous *N. lugens* is longer than that in brachypterous ones by about 1 day [37] because they have fully developed wings maintaining a great investment in flight apparatus [38]. Additionally, the reproductive success of *N. lugens* is also related to their ability to find a mate, mate, and search for food sources [23]. This study examined ovarian development by selecting only macropterous *N. lugens* and transferring them as pairs into separate mating tubes, which resulted in low competition. These differences could be attributed to the varying reproductive capacity of *N. lugens* in natural habitats compared to the results obtained from the laboratory. It is noticed that analyses of ovarian development and age distribution of key agricultural pests are important factors in understanding the fluctuation of field populations [20]. From this basic principle to practice in pest management, the ratio of the various ovarian age groups in the pest population is used to determine the reproductive status and indicate temporal-spatial population dynamics which can then be used to construct population models for the use of pest management actions [39]. Therefore, another important point of view to be discussed is a practical application of the criteria for predicting the emergence of *N. lugens* populations.

From an applied perspective, the classification of the ovary based on morphological criteria can be used to evaluate the population structure of the planthopper in field studies. There are pros to the morphological criteria conducted by Chen et al [19] and this study in the sense of application for pest control practices. Both criteria are helpful not only for the determination of ovarian development in *N. lugens*, but also for the expression of the simplistic form of population structure which is an important aspect of fieldwork. Much work was done by Hu et al [20], Zheng et al [21], and Ma et al [22] on migratory behaviors associated with reproductive development in *N. lugens* by using the criterion from Chen et al to categorize the *N. lugens* into 3 population types: (1) immigration; (2) part-emigration, part-sedentary, and local breeding; and (3) emigration. Whilst the central idea of our criteria emerges from the principle that ovarian changes are frequently accompanied by well-marked alterations in the morphological appearance and behavior of the animal [40]. Thus, the field population can be categorized into 3 groups related to the reproductive status and behavior of the *N. lugens* females including pre-ovulation, ovulation and oviposition, and post-oviposition.

The suitability of specimen types for each criterion is another issue to be discussed. A preservation method with ethyl alcohol has long been used in several countries for future analysis of pest infestation and prediction, but some disadvantages of alcohol-based fixatives include increases in viscosity, tissue shrinkage, and hardening. Whilst the oogenesis appears to be a continuous process, determination of ovarian development will suit the use with freshly killed insects if there are a lot of stages contained in the criteria. For this reason, the criterion from Chen et al is strongly recommended to be performed on newly dead insects. By comparison, the alternative criterion in this study offers a procedure that could be performed on both recently dead and preserved specimens as there are only 3 main phases of ovarian classification with few morphological features to recognize. In addition, this criterion might be useful when there is a shortage in the budget because of labor expense reduction.

In summary, there have been few previous studies that have combined investigation of the morphology

and physiology of the reproductive organs of insects corresponding to their oviposition behavior to establish criteria for determining field populations, especially about the major insect pests in rice agroecosystems. Thus, the simple morphological criteria gained from this study provide a basis for identifying the phase of the ovary for translating such physiological data to reproductive age groups, allowing us to predict the phenology of *N. lugens* populations in rice fields and make pest control decisions. The researchers can use these criteria to estimate the occurrence of *N. lugens* offspring by randomly collecting *N. lugens* adults in the paddy fields at each rice growing phase. However, one limitation of our study is that the experiments were conducted under controlled conditions, whereas delayed mating could be caused by other environmental factors. An emerging important question is how accurate the degree of data is to predict changes in *N. lugens* populations under natural conditions. Therefore, future studies are necessary to confirm the accuracy of these criteria and their practical application in the field.

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