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Determination of instars of *Bactrocera dorsalis* (Diptera: Tephritidae)

Yan Shi[†], Lei Wang[†], Wei Dou[†], Hong-Bo Jiang[†], Dan-Dan Wei[†], Dong Wei[†], Jin-Zhi Niu[†], and Jin-Jun Wang^{†,*}

Abstract

The oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), is one of the most important economic pests in tropical and subtropical areas of the world, where it causes serious damage to fruit production. This study aimed to investigate the criteria for dividing the instars of *B. dorsalis*, which will be fundamental for the control and forecasting of development rates, as well as the development of efficient control measures for *B. dorsalis*. Five morphological variables, including the body length, the length and width of mouth hooks, and the length and width of the pharyngeal sclerite of the larvae, were measured. The Crosby growth rule was used in determining that *B. dorsalis* has 3 instars. The length of the pharyngeal sclerite is the best morphological variable for distinguishing the instars of *B. dorsalis*, whereas the length and the width of mouth hooks and the width of the pharyngeal sclerite can be used as additional characteristics. There was an overlap in the body length between the adjacent instars of *B. dorsalis*, and therefore body length cannot be used to separate the instars accurately.

Key Words: oriental fruit fly; instar; sclerite structure

Resumen

La mosca de la fruta oriental, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), es considerada una de las plagas más importantes desde el punto de vista económico en las zonas tropicales y subtropicales del mundo, donde causa daños serios a la producción de frutas. El objetivo de este estudio fue investigar el criterio para dividir los instares de *B. dorsalis*, que serán fundamentales para el control y pronóstico de la tasa de desarrollo, así como el desarrollo de medidas de control eficaces contra *B. dorsalis*. Se midieron cinco variables morfológicas, incluyendo la longitud corporal, la longitud y el ancho de los ganchos bucales y la longitud y el ancho de las escleritas faríngeas de las larvas. Se utilizó La regla de crecimiento de Crosby para determinar que *B. dorsalis* tiene 3 estadios. La longitud de las escleritas faríngeas es la mejor variable morfológica para distinguir los instares de *B. dorsalis*, mientras que la longitud y el ancho de los ganchos bucales y el ancho de las escleritas faríngeas pueden usarse como características adicionales. Hay una superposición en la longitud del cuerpo entre los instares adyacentes de *B. dorsalis*, por lo tanto, la longitud del cuerpo no se puede utilizar para separar los instars con precisión.

Palabras Clave: mosca de la fruta oriental; Instar; estructura de esclerita

The instars of insects are commonly separated based on the frequency distribution of body dimensions, particularly the width of the head capsule (Chen & Seybold 2013). In previous studies, researchers generally used histograms to estimate measurements. The number of instars was determined by the number of peaks in the histogram, and class limits for each instar were determined by visual inspection, followed by the application of the Brooks (Dyar) rule and the Crosby growth rule to verify the results (Crosby 1973; Loerch & Cameron 1983; Deng et al. 2015). Commonly, however, there are overlaps in the measurement distribution between adjacent instars (Cen et al. 2015; Deng et al. 2015; Li et al. 2015). In such situations, it is difficult to estimate the number of instar stages because the peaks of successive instars blend together in the histogram, and the boundaries between instars are indistinct.

The oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), is one of the most damaging and widespread economic pests in agricultural systems worldwide. It is able to attack over 250 host plants, including many commercial fruits, such as carambola, citrus, mandarin, and mango, as well as a large variety of agricultural products such as coffee and chili pepper (Jin et al. 2011; Hsu et al. 2014).

Studies found that much of the damage that this pest inflicts occurs through oviposition punctures and subsequent larval development in fruits (Hsu et al. 2014), which can easily destroy the marketability of fruits and cause huge economic losses. However, no research on the separation of instars of *B. dorsalis* has been reported. Determination of instars is important for pest control and entomological studies. For example, determination of instars can be used to estimate development times and predict when oviposition occurs, which can be incorporated into timing of management measures. It could also be used to measure the quality of various host fruits, using development rate of larvae as a criterion, which could contribute to our understanding of how fly species evolved to utilize plants across taxa.

Several reports on instar determination for Diptera species are available (Petitt 1990; Richardi et al. 2013; Cao et al. 2014; Cen et al. 2015; Deng et al. 2015). Measurement of either mouth hooks or the pharyngeal sclerite showed clear separation between adjacent instars of *Liriomyza sativae* Blanchard (Diptera: Agromyzidae) (Petitt 1990). Morphological characteristics of the larval pharyngeal sclerite of horn flies and a leafminer can be used to identify larval instars (Cao et al. 2014; Deng et

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al. 2015). In *Austrosimulium tilyardianum* Dumbleton (Diptera: Simuliidae), larvae were separated into 9 instars based on head capsule width or head capsule length (Cen et al. 2015). Morphometry of the antennae, mandibles, mentum, and ventromental plates was used to discriminate among the 4 instars of *Chironomus sancticarloi* Strixino & Strixino (Diptera: Chironomidae) (Richardi et al. 2013). The objective of this study was to find a scientific, accurate, simple, and fast method that can be used as a classification criterion of instars of *B. dorsalis*, and we measured larval body length, the length and width of mouth hooks, and the length and width of the pharyngeal sclerite of *B. dorsalis*.

Materials and Methods

INSECTS

The laboratory colony of *B. dorsalis* (taxon identifier: 27457) was originally collected from fields in Hainan, China, in 2008. To obtain the required number of individuals, newly emerged larvae were fed an artificial diet consisting of corn flour, wheat germ flour, yeast powder, agar, sugar, sorbic acid, vitamin C, and linoleic acid on filter paper. After eclosing, the adults were fed on an artificial diet consisting of yeast powder, honey, sugar, vitamin C, and water in rearing boxes (Wang et al. 2013). All life stages of *B. dorsalis* were reared in a temperature-controlled incubator at 27 ± 0.5 °C and $70 \pm 5\%$ relative humidity with a photoperiod of 14:10 h L:D.

LARVAE COLLECTION AND MEASUREMENTS

Eggs were collected from *B. dorsalis* females by inducing oviposition through perforated parafilm coated with orange juice for 1 h, then placed on moist fabric and maintained at 27 ± 0.5 °C and $70 \pm 5\%$ relative humidity. The first batch of larvae was collected after 36 h. Afterwards, larvae were collected once every 8 h with 3 collections conducted in 1 d. Thirty larvae were collected each time, with 20 larvae used for measurements and 10 preserved as voucher specimens. All larvae were preserved in 70% alcohol. The examined specimens were macerated in 10% NaOH and measured in glycerin jelly with a Leica® M205A stereomicroscope (Leica Microsystems Ltd., Wetzlar, Germany). All drawings were made with the aid of Adobe® Photoshop® CS5 software (Adobe, San Jose, California). Voucher specimens were deposited in the insect collection of Southwest University, Chongqing, China.

STATISTICAL ANALYSES

All statistical analyses were performed with SPSS® 20.0 for Windows® (IBM, Chicago, Illinois), and graphs were plotted with Microsoft® Office Excel 2016 for Windows® (Microsoft Corporation, Redmond, Washington). Based on the larval body length, the length and width of mouth hooks, and the length and width of the pharyngeal sclerite of *B. dorsalis* (Fig. 1), a frequency distribution analysis was performed and a histogram of frequency distribution for each measured variable was drawn. Subsequently, the mean, standard deviation, and

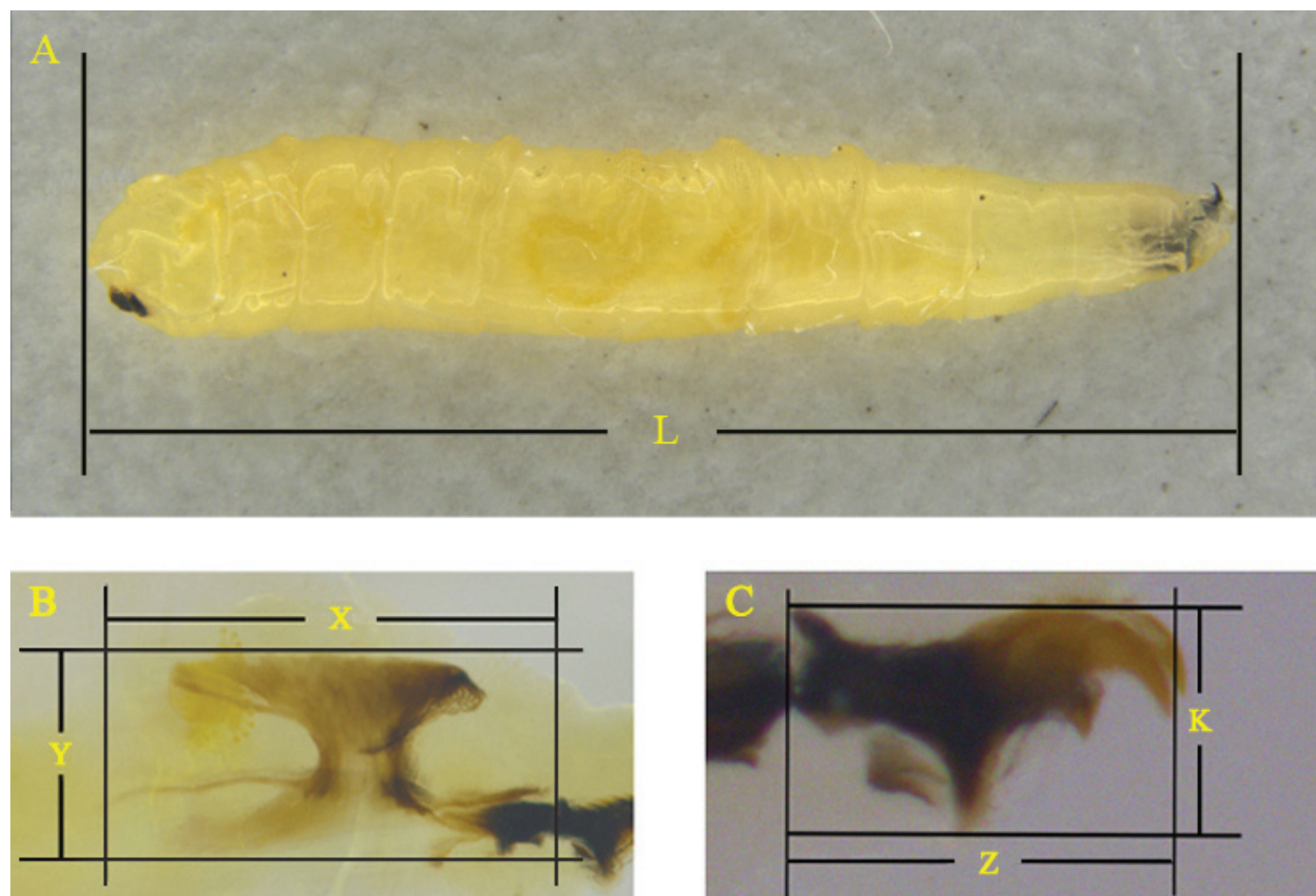


Fig. 1. Schematic diagram of the measurements for *Bactrocera dorsalis* larvae. A: Body length of *B. dorsalis* larvae (L); B: pharyngeal sclerite length (X) and width (Y); C: mouth hook length (Z) and width (K).

coefficient of variation for each variable were computed. Combined with the Crosby growth rule and regression analysis, the differences in the 5 morphological variables between successive instars were compared, and the best criteria of instar division were determined (Crosby 1973). When the value of the Crosby index is greater than 10%, it shows that it is unreasonable to determine instars by this variable (Loerch & Cameron 1983). The formula is as follows:

$$\text{Brooks index} = X_n - X_{n-1}; \text{Crosby index} = (b_n - b_{n-1}) / b_{n-1}$$

In the formula, X_n and X_{n-1} represent the average values of n and $n-1$ instar for each variable; b_n and b_{n-1} represent the Brooks index of n and $n-1$ instar, respectively.

Results

DETERMINATION OF THE CRITERIA FOR DIVIDING THE INSTARS OF *B. DORSALIS*

In total, 321 individual larvae were used for the measurements. For each morphological variable, we performed a frequency distribution analysis and drew a histogram. A concentrated area of frequency distribution represented an instar. The results showed that the length and width of mouth hooks and of the pharyngeal sclerite exhibited 3 distinct distribution areas (Fig. 2B–E). In contrast, the larval body length was concentrated in a large area, which could be divided into only 2 undefined areas (Fig. 2A). Based on pairwise combinations of 4 variables (the length and width of mouth hooks and of the pharyngeal sclerite), we drew scatter plot distributions (Fig. 2F–K). Generally, morphological measurements from enough samples are scattered normally, hence the peaks likely represented distinct instars. Combined with the histogram and scatter plot, the results suggested that the larvae of *B. dorsalis* can be divided into 3 instars.

Based on the mean, range, standard deviation, coefficient of variation, in combination with Brooks index and Crosby index, of the 5 measured variables (Table 1), our results indicated a significant difference between the mean of the 5 variables between instars ($P < 0.01$). There was no overlap in the length and width of the pharyngeal sclerite and mouth hooks between instars of *B. dorsalis*. Thus, these 4 morphological features are useful variables for the division of the instars. There was an overlap in the range of body length between the adjacent instars of *B. dorsalis*, wherefore this variable cannot be used to separate the instars accurately.

THE MATHEMATICAL RELATIONSHIP BETWEEN THE CRITERIA OF INSTAR DIVISION AND INSTARS

In previous studies, linear and modified exponential relationships between the logarithm of the length for skeleton structures and instars were determined (Chen & Pang 1988; Chowdhury et al. 2009). Using SPSS® 20.0 software, we performed linear and exponential regression analyses using the logarithm (\log_{10}) of the 5 variables for instars. The results indicated that there was a significant correlation between the logarithm of the length and width of mouth hooks and of the pharyngeal sclerite and instars. However, a poor correlation existed between the logarithm of larval body length and instars (Table 2). These results reconfirmed that it was reasonable to determine the instars of *B. dorsalis* by the length and width of mouth hooks and of the pharyngeal sclerite, and verified that the body length cannot be used to separate the instars accurately.

The results for the 5 variables showed that the coefficients of determination (R^2) of the regression equations were high. The R^2 was highest for the length of the pharyngeal sclerite and instars in all fitted models (cubic equation: $R^2 = 0.993$; quadratic equation: $R^2 = 0.992$; exponential equation: $R^2 = 0.991$). Therefore, the length of the pharyngeal sclerite is better to use than the length of mouth hooks for dividing the instars. The 3 variables of width of pharyngeal sclerite, width of mouth hooks, and length of mouth hooks can be used as auxiliary and verification standards.

MORPHOLOGICAL FEATURES OF EACH INSTAR OF *B. DORSALIS*

The duration of the 1st instar at 27 °C was 2 d. The pharyngeal sclerite was developing and lightly sclerotized with a stick-like shape. The mouth hooks were tiny, symmetrical, and lightly sclerotized. Apically, each mouth hook formed a conspicuously down-curved, sharply pointed hook. A pair of posterior spiracles had a columnar shape. However, it was difficult to see the anterior spiracles (Fig. 3A, B).

The duration of the 2nd instar at 27 °C was 2 d. The pharyngeal sclerite was further sclerotized with a more defined shape than that of the 1st instar. The mouth hooks were further sclerotized, and apically each mouth hook formed a conspicuous down-curved, sharply pointed hook. The 2 anterior spiracles were lobe-like shaped. The posterior spiracles were C-shaped and symmetrical, with a flat surface and incomplete annulus (Fig. 3C, D).

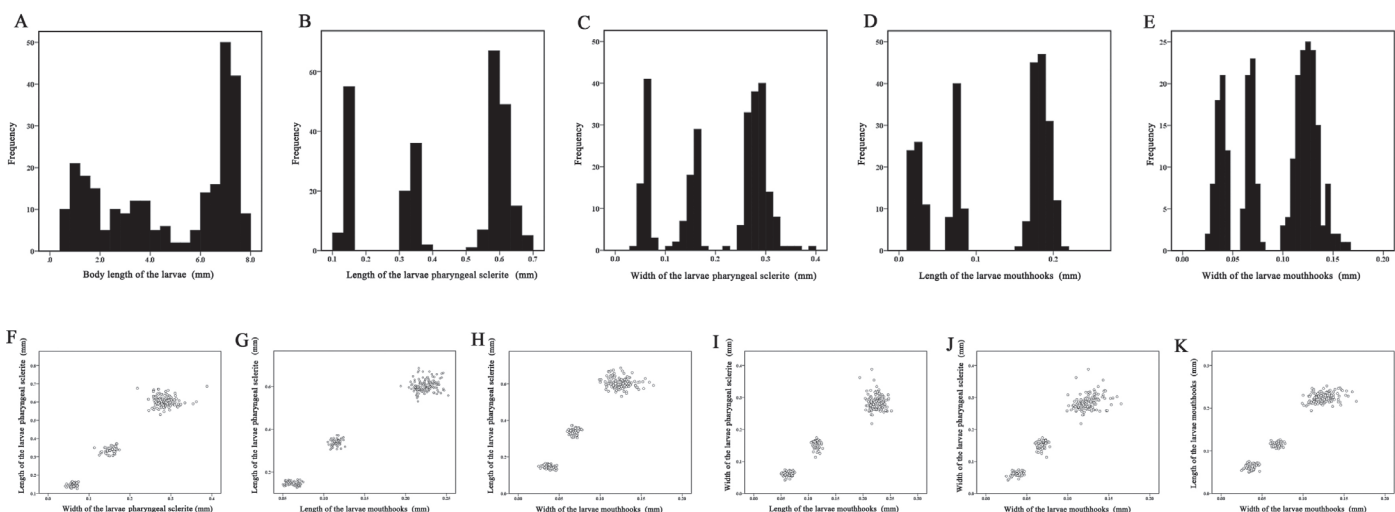


Fig. 2. Frequency histograms and scatter plots of measurements of 5 morphological variables for *Bactrocera dorsalis* larvae.

Table 1. Measurements and statistics of 5 morphological variables for the determination of instars of *Bactrocera dorsalis*.

Morphological variable	Instar	Sample size (n)	Mean \pm SE (mm)	Range (mm)	Variable coefficient	Brooks ratio	Crosby ratio
Body length	1st	61	1.2449 \pm 0.0532a	0.5640–2.0750	0.3335		
	2nd	58	3.1835 \pm 0.0945b	1.6470–4.5000	0.2261	2.5572	–0.1530
	3rd	144	6.8954 \pm 0.0579c	4.6290–7.9350	0.1007	2.1660	
Pharyngeal sclerite length	1st	61	0.1456 \pm 0.0012a	0.1260–0.1650	0.0654		
	2nd	58	0.3394 \pm 0.0020b	0.3050–0.3720	0.0446	2.3307	–0.2370
	3rd	144	0.6036 \pm 0.0023c	0.5310–0.6860	0.0458	1.7784	
Pharyngeal sclerite width	1st	61	0.0613 \pm 0.0009a	0.0420–0.0740	0.1110		
	2nd	58	0.1538 \pm 0.0016b	0.1130–0.1740	0.0803	2.5070	–0.2612
	3rd	144	0.2849 \pm 0.0019c	0.2180–0.3880	0.0795	1.8522	
Mouth hook length	1st	61	0.0626 \pm 0.0009a	0.0500–0.0750	0.1094		
	2nd	58	0.1151 \pm 0.0006b	0.1050–0.1260	0.0402	1.8393	0.0589
	3rd	144	0.2242 \pm 0.0009c	0.1940–0.2520	0.0487	1.9475	
Mouth hook width	1st	61	0.0379 \pm 0.0007a	0.0250–0.0470	0.1355		
	2nd	58	0.0683 \pm 0.0006b	0.0600–0.0780	0.0619	1.8023	0.0162
	3rd	144	0.1251 \pm 0.0010c	0.0990–0.1650	0.0968	1.8315	

Data are mean \pm SE, and different lowercase letters following the data indicate significant difference in the same variable between instars of *B. dorsalis* at the 0.01 level by Fisher LSD test.

The duration of the 3rd instar at 27 °C was 6 d. The pharyngeal sclerite was well sclerotized. The mouth hooks were massive, symmetrical, and strongly sclerotized. The apical part of each mouth hook had the form of a down-curved, pointed hook, which was lighter in curvature than in the other instars. Each hook was considerably thicker compared with counterparts in the other instars. The structures of anterior and posterior spiracles were similar to those in the 2nd instar (Fig. 3E, F).

Discussion

Although the bodies of larvae regularly grow and continually increase in length, the sizes of certain sclerotized body parts, such as the head capsule, mouthparts, antennae, and mandible, display discontinuous growth rates because the growth of these sclerotized parts occurs only when an insect molts and a new, soft cuticle is produced and expanded (Chapman 1998). The process of molting is the most direct

and reliable way to observe the situation of larvae molting for dividing the instars of insects. However, it is difficult to observe the process of molting for insects that are hidden in fruit or other plant or animal tissues. Based on the Dyar rule, most researchers believe that the growth of sclerotized parts proceeds in a regular geometric progression between successive instars (Crosby 1973; Irigaray et al. 2006; Gómez et al. 2015). One of the most commonly used features is the width of the larval head capsule (Hammack et al. 2003; Delbac et al. 2010; Wu et al. 2012). Body length of most insects increases according to the rule that growth of body length is continuous under normal circumstances (Chen & Pang 1988; Deng et al. 2015); therefore, we can only roughly estimate larval instar by the body length.

The pharyngeal sclerite is a sclerotized structure of fly larvae, which has often been used as a morphological variable to divide the instars. Many studies have focused on the instar division of Tephritidae, Agromyzidae, and Muscidae (Petitt 1990; Cao et al. 2014; Deng et al. 2015). The measurements of the pharyngeal sclerite and mouth hooks are often used as characteristic indicators of larval instars. Our results

Table 2. Regression between instar numbers and morphological variables for the determination of the number of instars of *Bactrocera dorsalis*.

Morphological variable	Fitted model	Regression equation	Coefficient of determination
Body length	Linear	$y = 2.392x - 0.924$	0.909
	Quadratic	$y = 1.486x^2 + 1.107x + 1.008$	0.937
	Cubic	$y = -2.804x^3 + 4.535x^2 + 0.631x + 0.913$	0.946
	Exponential	$y = 1.006e^{1.287x}$	0.905
Pharyngeal sclerite length	Linear	$y = 3.247x + 3.673$	0.984
	Quadratic	$y = 1.845x^2 + 5.168x + 4.037$	0.992
	Cubic	$y = -1.252x^3 + 4.389x + 3.943$	0.992
	Exponential	$y = 4.434e^{1.758x}$	0.991
Pharyngeal sclerite width	Linear	$y = 2.966x + 4.572$	0.971
	Quadratic	$y = 1.437x^2 + 5.476x + 5.542$	0.979
	Cubic	$y = -0.590x^3 + 4.387x + 5.282$	0.980
	Exponential	$y = 7.216e^{1.606x}$	0.980
Mouth hook length	Linear	$y = 3.524x + 5.285$	0.984
	Quadratic	$y = 0.145x^2 + 3.790x + 5.398$	0.984
	Cubic	$y = -0.091x^3 + 3.760x + 5.417$	0.984
	Exponential	$y = 10.359e^{1.879x}$	0.962
Mouth hook width	Linear	$y = 3.685x + 6.307$	0.960
	Quadratic	$y = 0.046x^2 + 3.792x + 6.366$	0.960
	Cubic	$y = -0.070x^3 + 3.972x + 6.517$	0.960
	Exponential	$y = 17.950e^{1.969x}$	0.943

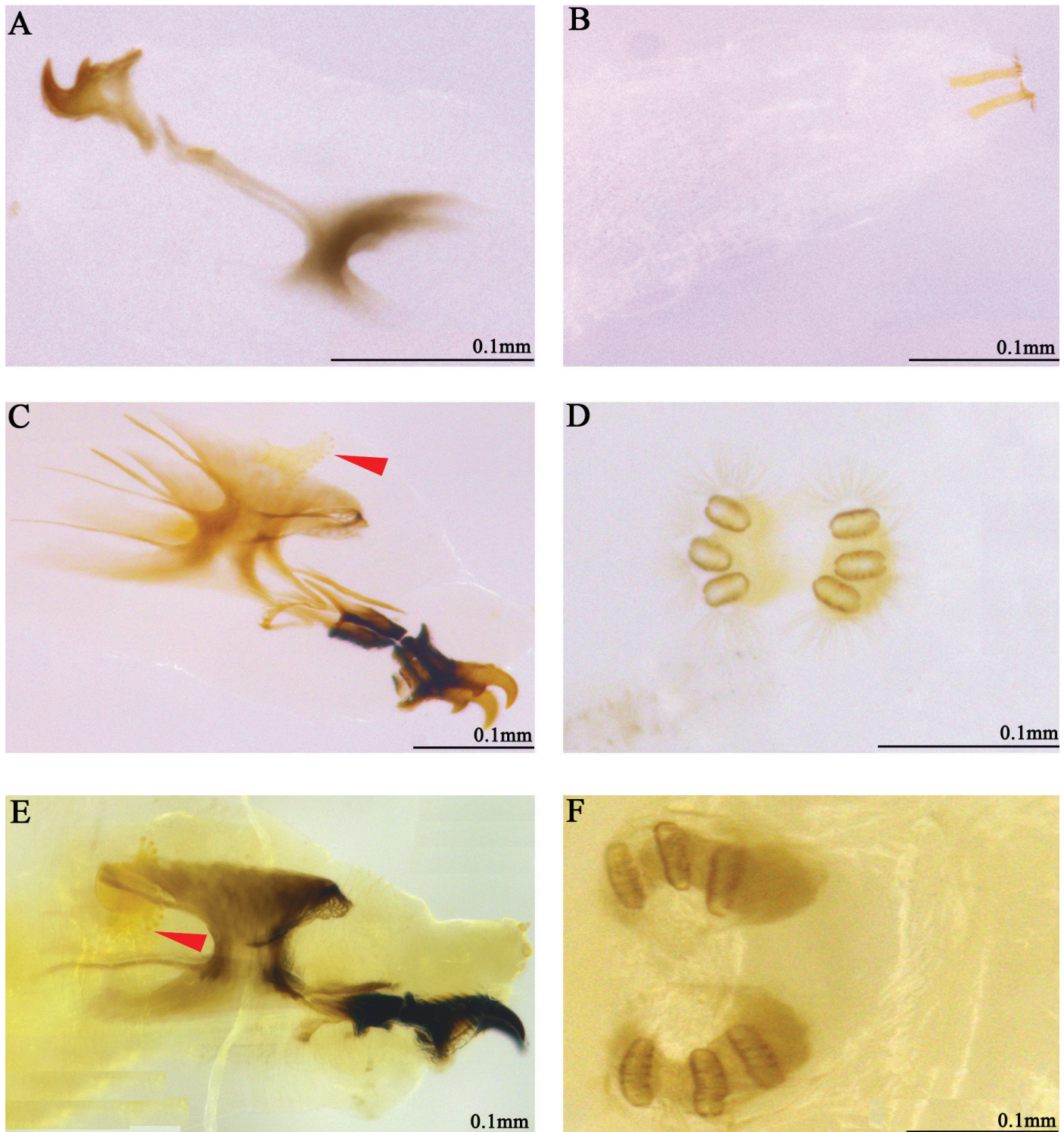


Fig. 3. Morphological characteristics of the pharyngeal sclerite, mouth hooks, anterior spiracles, and posterior spiracles of *Bactrocera dorsalis* larvae in three instars. A, C, E: Pharyngeal sclerite and mouth hook of the 1st, 2nd, and 3rd instar, respectively; B, D, F: posterior spiracles of the 1st, 2nd, and 3rd instar, respectively; red arrows indicate the anterior spiracles of the 2nd and 3rd instar in C and E, respectively.

suggested that the larvae of *B. dorsalis* can be divided into 3 instars. Because of the body length amplitude overlapping between adjacent instars, the body length cannot be applied to accurately determine an instar. However, the body length of adjacent instars did significantly differ ($P < 0.01$). The morphological characteristics of the mouth hooks, pharyngeal sclerite, anterior spiracles, and posterior spiracles are

key features for dividing the instars of a sarcophagid flesh fly (Vairo et al. 2015). For determination of the instars of *B. dorsalis*, we have described the morphology of the pharyngeal sclerite, mouth hooks, anterior spiracles, and posterior spiracles in this research. The results indicate that we can quickly determine the instar by these morphological characteristics.

Acknowledgments

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