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Insights into pupal development of *Bactrocera dorsalis*: factors influencing eclosion

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In the life cycle of the oriental fruit fly where larvae reside within fruits and adults exhibit high activity, the pupal stage occurs in the soil, closely tied to agricultural soil management. This study investigates the impact of four variables (body orientation, burial depth, soil particle size, and pH) on *Bactrocera dorsalis*' physiological preferences, eclosion rates, and pupal stage duration. Notably, body orientation, burial depth, soil texture (particle size), and pH affect eclosion rates. The fruit fly demonstrates a preference for a supine position during eclosion, with average eclosion rates of 97.33%, contrasting with decreases to 78.00% in a head-down position. Soil with mixed particle sizes is detrimental to pupal eclosion, reducing the eclosion rate to 56.67%. And at a pH of 9.98, eclosion rates decrease to 16.67%. These factors significantly influence eclosion rates However, these factors do not significantly alter the duration of the pupal stage, with mean changes not exceeding one day when experimental conditions are modified. These findings suggests that soil manipulations affecting these variables could reduce eclosion rates without compromising the uniformity of adult emergence. This study provides a foundation for environmentally friendly pest control practices and addresses gaps in the pupal stage research of the oriental fruit fly.

Keywords Eclosion, Pupa stage, Position, Soil modification, Soil type

The pupal stage of insects is characterized by the encapsulation of insects, a period of dormancy without feeding or movement, and intricate metamorphic development[1]. During this phase, insects undergo crucial morphological transformations and organ development essential for their subsequent transition to the adult sta ge[2345678910111213]. The inherent immobility of insects during pupation exposes them to heightened vulnerability, both from natural predators and human interventions. This pivotal phase attracts attention in entomological studies due to its scientific significance and practical implications.

In pupal stage research, a primary focus often revolves around factors associated with the emergence of adults, such as the eclosion rate and duration of pupal stage, which are intricately linked to the quantity and uniformity of adult emergence[5,8,11,12]. Presently, the majority of studies investigating these aspects focus on variations induced by temperature, humidity, and light conditions[456711121314]. However, research on the impact of other factors is currently relatively scarce, indicating a need for further exploration in this area.

The oriental fruit fly (*Bactrocera dorsalis*) is a globally significant pest, capable of infesting multiple host species[15161718]. Its larvae develop inside fruits[19], while the adults exhibit strong mobility, allowing them to traverse various fruit-producing regions, which posing substantial challenges for control efforts[20,21]. Given the variation in soil conditions across different fruit-growing areas and the fact that the pupal stage of *B. dorsalis* occurs in the soil, exploring the agricultural practices and soil conditions that may affect the pupal stage can provide novel insights and methods for controlling this pest. Such research offers new perspectives and enriches our understanding of this species.

In our study, we assessed the variation in eclosion rates of the oriental fruit fly (*B. dorsalis*) under different head orientations, soil particle sizes, soil pH levels, and burial depths. Additionally, we investigated the changes in the pupal stage duration under these varied conditions. Our objective was to understand the most suitable conditions for development of the oriental fruit fly and the impact of different soil treatments on its eventual eclosion into an adult. This research provides new insights for integrated pest management for the oriental fruit fly.

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Results

Physiological preferences of head position in *B. Dorsalis*

The positioning of the *B. dorsalis* head during pupation influences the emergence rate and exhibits a discernible positional preference (Fig. 1). Specifically, the highest average emergence rate was observed in the 'lie' position, followed by '45º up', then '45º down' and 'up'. The 'down' position showed a significantly lower emergence rate compared to both the 'lie' and '45º up' positions (F = 3.820, df = 4, 10, $p = 0.039$) (Fig. 1).

Eclosion depth trend in *B. Dorsalis*: favorability of intermediate depths

The influence of various pupation depths on the eclosion rate of *B. dorsalis*was statistically significant (LRT: $\chi^2(4) = 75.127$, $p < 0.001$); We conducted the analysis using binomial logistic regression and assessed the statistical significance with a likelihood ratio test (LRT). In the pairwise comparisons of different pupation depths, the eclosion rate at a 5 cm depth was significantly higher than at 1 cm and 9 cm depths (Tukey HSD: depth 1 cm vs. depth 5 cm: $p = 0.0142$; depth 5 cm vs. depth 9 cm: $p < 0.001$). There was no significant difference between the 3 cm, 5 cm and 7 cm depths (Tukey HSD: depth 3 cm vs. depth 5 cm: $p = 0.6915$; depth 3 cm vs. depth 7 cm: $p = 0.8378$; depth 5 cm vs. depth 7 cm: $p = 0.1768$). The eclosion rate at 1 cm depth was significantly lower than at 5 cm but higher than at 9 cm (Tukey HSD: depth 1 cm vs. depth 5 cm: $p = 0.0142$; depth 1 cm vs. depth 9 cm: $p = 0.0003$). The eclosion rate at a 9 cm depth was significantly lower than at other depths (Tukey HSD: $p < 0.0001$). Both deep (9 cm) depths and the shallow(1 cm) depth were not conducive to the eclosion of the *B. dorsalis*, whereas intermediate depths (3 cm, 5 cm) were more favorable for eclosion (Fig. 2). A noticeable trend was observed, with a decrease in eclosion rate at both shallow and deep depths (Fig. 2). Additionally, increased variability was evident with greater depth; in the 9 cm treatment, the worst-performing replicate had only 34% successful emergence, while the best-performing replicate had an 88% successful emergence. (Fig. 2).

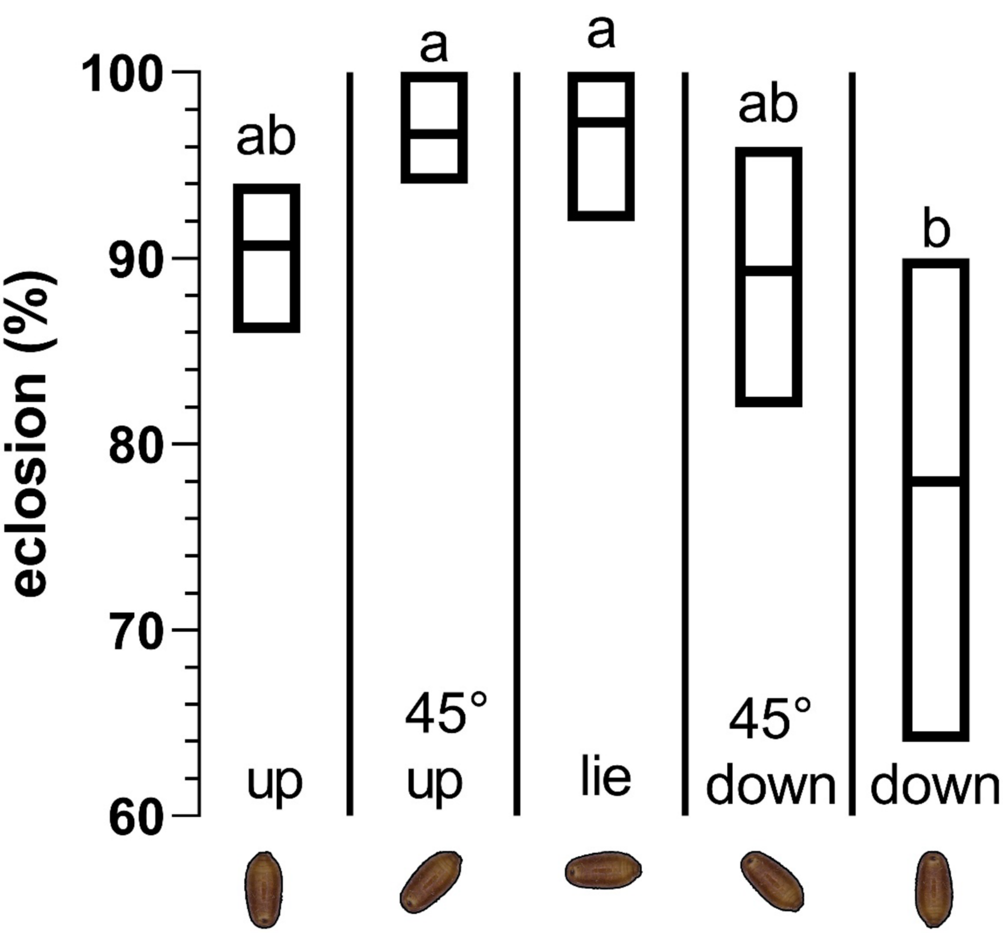

Fig. 1. Impact of head orientation during pupation on the eclosion rate of *B. dorsalis*. On the X-axis, moving from left to right, the head orientations include head up, inclined upward at 45°, lie, inclined downward at 45°, and head down. In this box plot, the central line represents the mean eclosion rate of the insects, while the box extends from the bottom to the top, indicating the range from the minimum to the maximum values. Lowercase letters above boxes denote significant differences between different treatments (one-way analysis of variance, LSD post-hoc test).

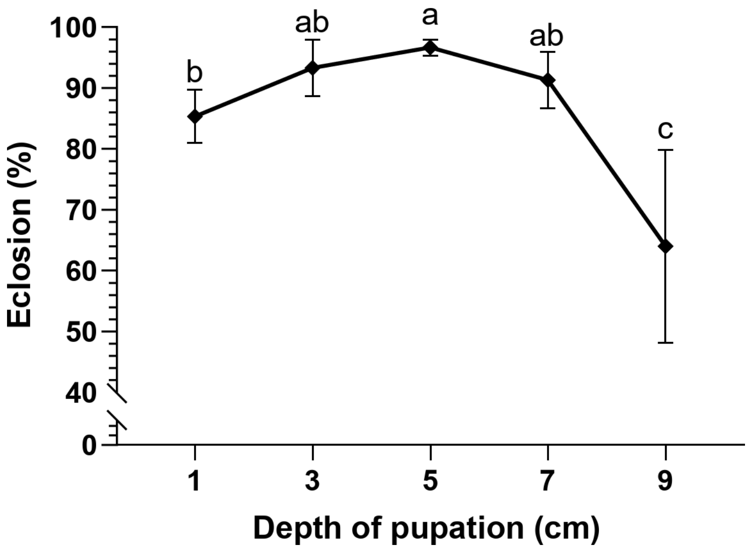

Fig. 2. Mean (± SEM) percentage eclosion of *B. dorsalis* at different pupation depths. All experiments were conducted using the "lie" position. Points represent the mean eclosion percentage, and error bars indicate the standard error of the mean (SEM). Lowercase letters on the error bars denote significant differences between different treatments (Tukey HSD test: $p<0.05$).

	Eclosion (%)		
Texture	Mean	Min.	Max.
Sandy soil	90.67 ± 2.91 a	86.00	96.00
Loam	56.67 ± 3.71 b	52.00	64.00
Clay	96.00 ± 4.00 a	88.00	100.00

Table 1. The emergence ratio of *B. Dorsalis* in different soil textures. Note: Soil classification adhered to the "International standard for soil texture classification" and was determined using the specific gravity method. The data in the table represent the mean ± standard error, with lowercase letters in the table denoting significant differences between different treatments (Tukey HSD test: $p<0.05$).

Mixed particle size soil hinders eclosion

Different soil types, distinguished by their particle size compositions, demonstrate a notable impact on the eclosion of *B. dorsalis*(LRT: $\chi^2(4)=87.439$, $p<0.001$) (Table 1); Specifically, the eclosion rate significantly decreases in loam soils (Table 1), characterized by a relatively equal distribution of medium-sized (0.002 ~ 0.02 mm) and larger particles (0.02 ~ 2 mm). In contrast, the eclosion rate is higher in soils with either larger (0.02 ~ 2 mm) or smaller (< 0.002 mm) particle sizes, such as sandy soil and clay soil (Table 1) (Tukey HSD: loam vs. sandy soil: $p<0.0001$; loam vs.: clay: $p<0.0001$).

Soil pH tolerance and eclosion rate of *B. Dorsalis*

Changes in soil acidity and alkalinity can lead to variations in the eclosion rate of *B. dorsalis*. Notably, as the pH approaches 8.38, the eclosion rate exhibits higher variation, suggesting a potential influence of soil pH on *B. dorsalis* pupae (Fig. 3). Furthermore, there is a significant decrease in the eclosion rate when the pH reaches 9.98 (F = 4.826, df = 4, 10, $p=0.02$) (Fig. 3). In contrast, when the soil becomes more acidic, the impact on the eclosion rate of *B. dorsalis* pupae is not substantial, indicating a possible higher tolerance of *B. dorsalis* pupae to acidity (Fig. 3).

No significant effect on pupal stage duration

Throughout the pupal stage, none of the treatments—head orientation (Kruskal–Wallis test: df = 4, $p=0.120$), pupation depth (Kruskal–Wallis test: df = 4, $p=0.985$), soil texture (particle size) (Kruskal–Wallis test: df = 2, $p=0.737$), and pH (Kruskal–Wallis test: df = 4, $p=0.277$) — demonstrated a significant effect on the pupal stage (Fig. 4). Although Fig. 4 presents some extreme individual values, the overall trend remains consistent, indicating that these values have a limited impact on the overall analysis of the pupal stage.

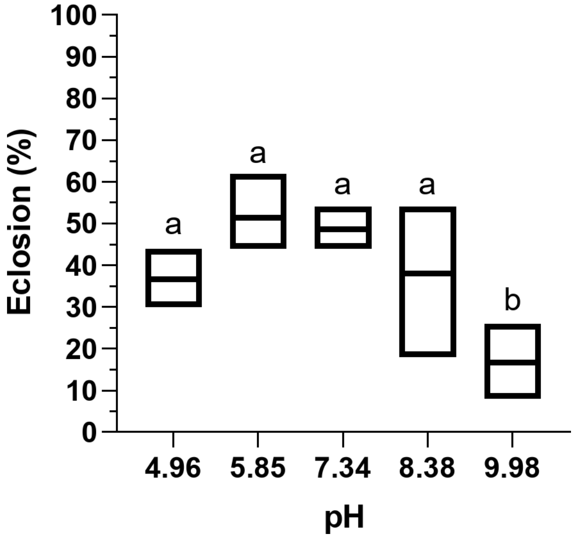

Fig. 3. Influence of soil pH on the eclosion rate of *B. dorsalis*. All experiments were conducted using the "lie" position, which yielded the highest eclosion rate. In this box plot, the central line represents the mean eclosion rate of the insects, while the box extends from the bottom to the top, indicating the range from the minimum to the maximum values. Lowercase letters above boxes denote significant differences between different treatments (one-way analysis of variance, LSD post-hoc test).

Discussion

In our experiments, we observed a significant decrease in the eclosion rate of *B. dorsalis* pupae when the head orientation was downward. Simultaneously, the 'lie' position showed the highest average eclosion rate; The eclosion rate in the 'head-down' position was significantly lower than in the 'head-up' and '45° up' positions. Although there was no statistically significant difference among all groups, there appeared to be a trend suggesting that positions closer to the 'lie' posture were more conducive to eclosion (Fig. 1). Previous studies on the pupal head orientation in different fly species have indicated variations in preferred positions. For instance, *Drosophila melanogaster* Meigen and *Drosophila funebris* Fabricius tend to favor head-up or close-to- 'lie' positions, while *Drosophila simulans* Sturtevant exhibits a preference for head-up or close-to-tilted-up at 45 degrees. *Zaprionus tuberculatus*Malloch, on the other hand, tends to favor head-down or close-to-prone positions[22]. The inclination of the oriental fruit fly, *B. dorsalis*, to adopt a 'lie' pupation position may be attributed to the potential advantage of facilitating the separation between its body and the pupal shell during eclosion. In a previous study, it was observed that, during the early stages of pupation in *B. dorsalis*, a bubble filled with air appeared in the central part of the dorsal region[8]. The presence of this bubble serves to alter the thickness of the puparium, facilitating the separation of the pupal body from its exoskeleton. The process of bubble formation within the pupa and its release between the pupal body and exoskeleton, is closely associated with the outward flipping of organs such as the head, legs, and wings[8,232425]. Furthermore, in the investigation of gravity-sensation genes in the oriental fruit fly, it was observed that the expression of splice variants of these genes varies during the pupal stage[26]. Since the gravity-sensing organs of the oriental fruit fly are located in the antennae, further research is needed to determine whether there are differences in gravity-sensation among different developmental stages of the oriental fruit fly[26].

In a wealth of literature and practical observations, a correlation between pupation depth and the eclosion rate in *B. dorsalis*has been established[5,27]. Practical observations of the eclosion process in flies reveal that flies use the head and cheek parts to squeeze through the pupal case and drill out during eclosion. This process requires friction from the surrounding sand, so the depth of pupal burial cannot be too shallow. Otherwise, there would be insufficient sand to provide the necessary friction. Conversely, it cannot be too deep either, as the gravitational force of the sand and the crawling distance to the surface both impact the eclosion rate[28]. In some fly species, given ample space, larvae tend to select an optimal pupation depth for eclosion[29]. Our experiments similarly reveal that *B. dorsalis* exhibits an optimal pupation depth, reflecting a discernible trend in pupation depth variation (Fig. 2). Our findings align with previous research[27], showing that the 3, 5, and 7 cm depths are optimal for successful eclosion of *B. dorsalis*, with 1 cm and 7 cm being secondary favorable zones. When the burial depth exceeds 7 cm, reaching 9 cm, the average eclosion success rate further decreases to 64% (Fig. 2). However, it is noteworthy that within the three replicates at this depth, the highest emergence rate was still 88%, while the lowest was 34%, suggesting that *B. dorsalis* can still achieve relatively high eclosion rates in deeper soil beyond 5 cm (Fig. 2). It may also be that burial to a greater soil depth is required to consistently mitigate the individual capabilities of *B. dorsalis*, as demonstrated by variations observed in some studies and numerous pest control guidelines; Recommendations often suggest burying at depths of 50 cm or more to more effectively suppress the emergence of *B. dorsalis*adults[30]. Additionally, upon eclosion from the pupal stage, *B. dorsalis*needs to complete wing expansion within 20 min; otherwise, wing hardening may adversely affect survival[8]. The depth of pupation and soil particle size could influence the time it takes for *B. dorsalis* to emerge from the pupa

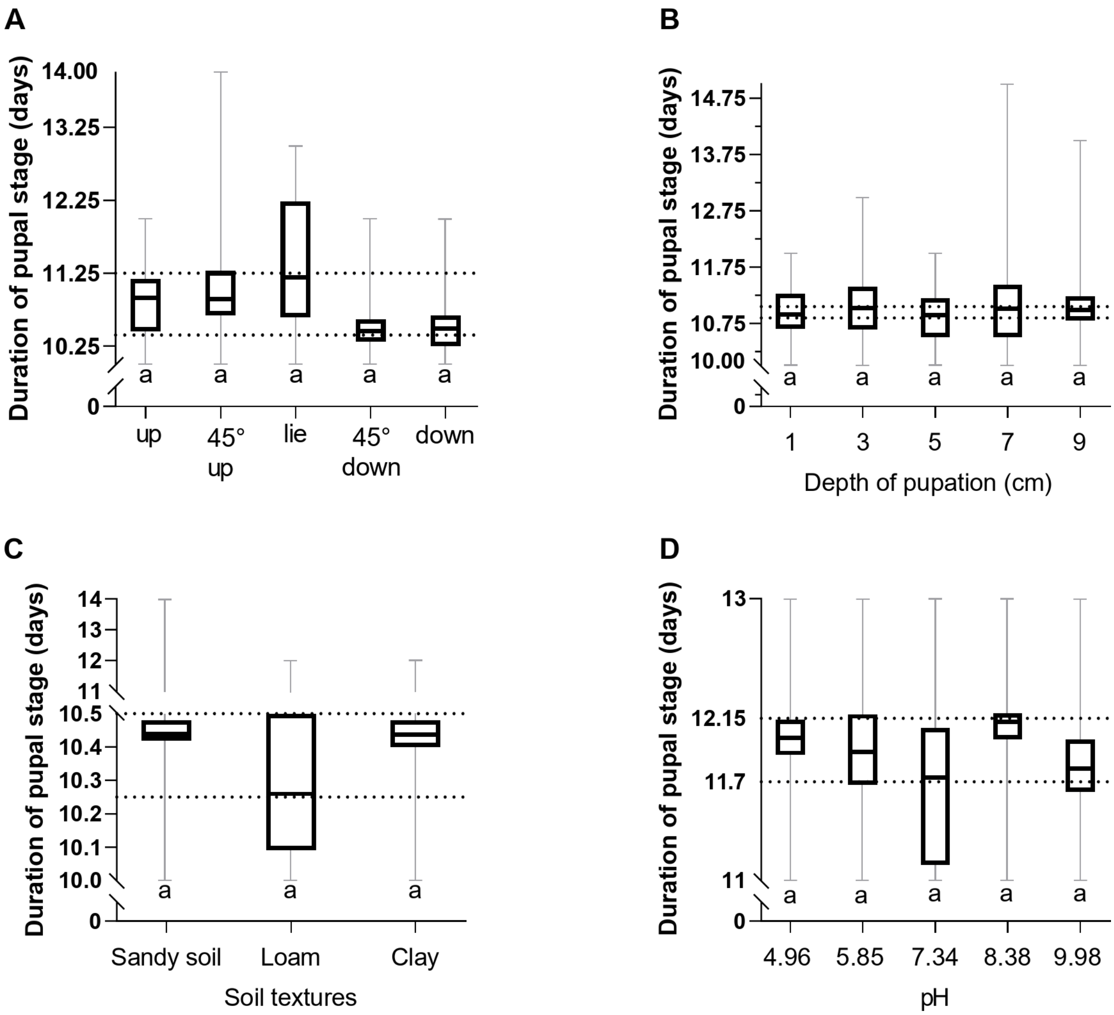

Fig. 4. Impact of Pupal Stage Environmental Changes on the Pupal Period of *B. dorsalis*. Soils used in Experiments A, B, and D were untreated natural soils. Experiments B, C, and D were conducted using the "lie" position, which yielded the highest eclosion rate. In this box plot, the central line represents the mean eclosion rate of the insects, while the box extends from the bottom to the top, indicating the range from the minimum to the maximum values of the mean values across biological replicates. Gray whiskers indicate the range of extreme individual values. Lowercase letters above boxes denote significant differences between different treatments (non-parametric test, Dunn's post-hoc test).

and reach the soil surface. Previous research on pupation depth indicates that, under conditions of sufficient soil moisture, *B. dorsalis* predominantly chooses depths of 4 cm or less for pupation. However, in situations of inadequate moisture, *B. dorsalis*tends to seek deeper soil depths for moisture availability[5].

The eclosion process in insects necessitates adequate friction, especially in dry soil conditions, where smaller soil particles contribute to increased external friction[31], while larger soil particles provide more surrounding space, impacting the eclosion process[32,33]. Previous studies on eclosion substrates have shown that in substrates composed of a mixture of particle sizes, and the eclosion rate is lower compared to substrates with single large or small particles. This could be attributed to the smaller particles in mixed substrates filling the spaces excavated by insects, making it more challenging for them to move in the soil. Larger particles require more force for insect movement, and the simultaneous presence of both may interfere with insect behavior in the soil[343536].Consistent with these findings, our study also reveals a significant decrease in the eclosion rate of *B. dorsalis* when exposed to mixed soil conditions with varying particle sizes (Table 1). Our research demonstrates that manipulating soil particles by altering their size can influence the eclosion of *B. dorsalis*, providing insights for pest control strategies through agricultural practices.

In our study, we observed an increase in individual variation in the eclosion rate of *B. dorsalis* as the pH reached 8.38, indicating that, by this point, pH begins to influence the pupae. At pH 9.98, there was a significant decrease in the eclosion rate. Conversely, as acidity intensified, the eclosion rate did not exhibit a significant change (Fig. 3). This observation suggests that *B. dorsalis* pupae demonstrate a stronger resistance to acidity compared to alkalinity. In the life history of *B. dorsalis*, instances arise where pupation occurs within fruit, potentially contributing to their heightened acid resistance[37]. Additionally, the larvae of *B. dorsalis*tend to modulate their living environment to a relatively stable acidity of around pH 4 before pupation, showcasing their ability to adapt to and modify their surroundings[38]. This aligns with our experimental results, where pH levels of 4.96 and 5.85 did not significantly affect the eclosion rate (Fig. 3). These findings underscore the potential efficacy of modifying soil acidity or alkalinity for *B. dorsalis* control. Alterations in acidity, particularly shifting from acidic to alkaline conditions, appear to exert a more substantial influence on *B. dorsalis*.

In our experiments, we observed that all treatments (head orientation, pupation depth, soil texture – particle size, and pH) did not have a significant impact on the duration of the pupal stage (Fig. 4). This suggests that the pupal stage of *B. dorsalis*is a relatively conservative and fixed period, with many factors not significantly affecting its duration. Insects capable of successful eclosion likely possess a stable biological clock, or the pupal stage is not easily influenced by these factors. In current research on Diptera, the pre-eclosion developmental time of fruit flies is primarily influenced by genetics and the duration of day and night[14,39], while there is currently a lack of studies in *B. dorsalis*. In the complex interplay between pupae and their natural environment, temperature, humidity and food seem to exert a more pronounced influence on the duration of the pupal stage compared to factors such as body position, depth, soil composition, and pH[5,404142]. Alternatively, it could be argued that the internal developmental timeline of the pupae is relatively fixed, and these factors such as body position, depth, soil composition, and pH impact whether pupae successfully complete their development and emerge as adults, without significantly affecting the duration of the developmental process. The fact that these treatments do not influence the eclosion time makes the approach of targeted pesticide application on newly emergence adults feasible. This alignment ensures that soil treatments and pesticide application targeting newly emergence adults are not conflicting, providing a novel perspective for the control of *B. dorsalis*.

Our study addresses gaps in the pupal stage research of *B. dorsalis*, shedding light on the impact of changes in pupal conditions and uncovering some physiological preferences of the oriental fruit fly. Additionally, we reveal that the duration of the pupal stage in *B. dorsalis* remains relatively constant and seems to be insignificantly influenced by factors such as body orientation, burial depth, soil texture (particle size), and pH. The prioritization of these influencing factors may represent a balance that *B. dorsalis* strikes in its interaction with natural conditions and predators.

Methods

Insect breeding and obtaining pupae

Bactrocera dorsalis were obtained from the orchard in Guangxi University (22.84 °N, 108.29 °E) and reared under controlled laboratory conditions, maintaining a constant temperature of 25 ± 1 ℃, relative humidity of $70 \pm 5\%$, and a photoperiod of 14 L:10D. Upon the initiation of pupation in wild larvae within mangoes, sand was provided as the pupation substrate. This sand was packed into a 5000 mL plastic box with a breathable mesh at the top. On the second day after the larvae pupated, a sample sieve (mesh size 50, diameter 30.0 cm, height 10.0 cm) was used to separate the newly formed pupae from the surrounding substrate. For each treatment, 150 *B. dorsalis* pupae were collected on the same day, with 50 pupae per replicate, and this process was systematically replicated in triplicate to ensure the robustness and reliability of data collection. In subsequent pupal experiments, the pupae developed under the same conditions as the larvae.

Evaluation of head orientation during the pupal stage on eclosion rate of *B. Dorsalis*

A total of 750 pupae *B. dorsalis* was classified into five distinct head orientations: head up, head down, lie, head leaning up 45 degrees, and head tilting down 45 degrees. For each head orientation, 150 pupae were used and randomly selected, with 50 pupae per replicate across three replicates. Pupae were subsequently buried at a consistent depth of 3 cm under the sand, with a 2 cm separation between adjacent pupae. This pupation depth control method was consistently applied in subsequent experiments. Initially, a 1 cm layer of sand was evenly spread at the bottom of the plastic box. Next, the pupa's head was positioned as specified, followed by covering it with 3 cm of sand using a sieve. The emergence date and the number of insects emerging were recorded until all pupae had completed eclosion.

Evaluation of soil depth during the pupal stage on eclosion rate of *B. Dorsalis*

A total of 750 pupae of *B. dorsalis* were used in the experiment. For each of the five soil depths (1 cm, 3.0 cm, 5.0 cm, 7.0 cm, and 9.0 cm), 150 pupae were randomly selected and buried at that specific depth, with 50 pupae per replicate across three replicates.The pupae were positioned in the "lie" orientation as per the methodology described in Section "**Evaluation of head orientation during the pupal stage on eclosion rate of*B. dorsalis***", ensuring consistent spacing between adjacent pupae and utilizing the same depth control method applied in the Section "**Evaluation of head orientation during the pupal stage on eclosion rate of*B. dorsalis***". The emergence date and the number of insects emerging were recorded daily until all pupae completed their emergence. This allowed for an assessment of how soil depth influences both the pupal stage and the emergence rate of *B. dorsalis*.

Assessment of soil texture during the pupal stage on eclosion rate of *B. Dorsalis*

The soil samples utilized in this study were sourced from the campus of Guangxi University. We collected soil samples and air-dried them at 25°C and 70% relative humidity for approximately 7 days, until the weight difference over two consecutive days was less than 0.1%, ensuring that the soil samples reached a stable moisture content and approximated naturally air-dried soil. Next, we passed the samples through a 2 mm sieve to remove large stones and obtain air-dried samples; From the candidate soils, 50 g samples were taken, and the particle size proportion was determined using a hydrometer method; The samples were suspended in a liquid with a dispersing agent, and the particle size distribution was calculated by measuring changes in suspension density over time. This process is detailed in ISO 11277:2020(E)[43]. classify the soil types, adhering to the "International standard for soil texture classification"[44,45]. For Sandy soil, the proportion of sand (0.02–2 mm) exceeded 85%, while the combined percentage of silt (0.002–0.02 mm) and clay (< 0.002 mm) was less than 15%. Loam soil comprised sand (0.02–2 mm) in the range of 45–55%, silt (0.002–0.02 mm) between 30% and 45%, and clay (< 0.002 mm) less than 15%. In Clay soil, the percentage of clay (< 0.002 mm) fell between 45% and 65%, with silt (0.002–0.02 mm) accounting for less than 35% and sand (0.02–2 mm) less than 55%.Finally, the resulting sandy soil, clay, and loam were used to test the pupal duration and emergence of *B. dorsalis*. A total of 450 pupae of *B. dorsalis* were used in the experiment. For each of the three soil textures, 150 pupae were randomly selected and buried in a 3 cm deep soil layer, with 50 pupae per replicate across three replicates. The positioning of pupae in the 'lie' orientation followed the methodology outlined in Section "**Evaluation of head orientation during the pupal stage on eclosion rate of*B. dorsalis***", maintaining consistent spacing between adjacent pupae and employing the same method to control the depth of pupae buried in the soil as in Section "**Evaluation of head orientation during the pupal stage on eclosion rate of*B. dorsalis***".

Impact of soil pH during the pupal stage on eclosion rate of *B. Dorsalis*

Hydrochloric acid and sodium hydroxide were introduced to the naturally collected soil from Guangxi University to modulate its pH values. After thorough stirring, a soil in-situ pH meter (Spectrum, U.S.A, No: IQ150) was employed to measure and record the pH values for future reference. The final adjustments resulted in treatments with pH values of 4.96, 5.85, 7.34, 8.38, and 9.98. These treated soils were separately used to bury pupae in a 3 cm deep soil layer, adopting the 'lie' positioning followed the methodology outlined in Section "**Evaluation of head orientation during the pupal stage on eclosion rate of*B. dorsalis***". The spacing between adjacent pupae and the method for controlling the depth of pupae buried in the soil remained consistent with the procedures outlined in Section "**Evaluation of head orientation during the pupal stage on eclosion rate of*B. dorsalis***". A total of 750 pupae of *B. dorsalis* were used in the experiment, with 150 pupae randomly selected for each pH treatment, with 50 pupae per replicate across three replicates.

Statistical analysis

All data analyses were performed using SPSS version 27.0 (SPSS Inc., Chicago, IL, USA) and R version 4.4.1. Data on the effect of soil pH on the eclosion rate of *B. dorsalis* were analyzed using one-way analysis of variance (ANOVA) followed by the Least Significant Difference (LSD) post-hoc test ($p < 0.05$). Data on head orientation and its effect on the eclosion rate of *B. dorsalis* underwent arcsine square root transformation, followed by one-way ANOVA with LSD post-hoc test ($p < 0.05$). Data on soil texture and pupation depth, which did not meet the normality assumption, were analyzed using a generalized linear model (GLM) with a binomial distribution in R, utilizing the lme4, emmeans, and multcomp packages[464748], and Tukey's Honestly Significant Difference (HSD) test for multiple comparisons ($p < 0.05$). The Kruskal-Wallis test were performed for environmental changes (head orientation, soil depth, soil texture, soil pH) affecting the pupal period of *B. dorsalis*, with Dunn's post-hoc multiple comparison test conducted using the FSA package in R[49] ($p < 0.05$).

Data availability

The data that support the findings of this study can be obtained from the corresponding author upon reasonable request.

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Author contributions
Y.D. and W.L. conceived and conducted the experiments. Q.C. and Y.D. validated the results. Q.C. analyzed the data and wrote the paper. W.L. and X.W. acquired funding. X.W., X.Z., and W.L. were responsible for reviewing, editing, and project administration. All authors have read and agreed to the published version of the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

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