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Study of the Pupation Preference and Emergence Success of Ectropis grisescens by Using Choice and No-Choice Bioassays

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TITLE:

Choice and No-Choice Bioassays to Study the Pupation Preference and Emergence Success of *Ectropis grisescens*

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KEYWORDS:

Soil-pupation behavior, tea looper, *Ectropis grisescens*, emergence success, preference, substrate effect, substrate type, moisture content, choice test, no-choice test, *Camellia sinensis*

SHORT ABSTRACT:

Here, we present a protocol to investigate the pupation preference of mature larvae of *Ectropis grisescens* in response to soil factors (*e.g.*, substrate type and moisture content) using choice bioassays. We also present a protocol of no-choice bioassays to determine the factors that affect the pupation behaviors and survivorship of *E. grisescens.*

LONG ABSTRACT:

Many insects live above the ground as larvae and adults and as pupate below the ground. Compared to the above-ground stages of their life cycles, less attention has been paid on how environmental factors affect these insects when they pupate within the soil. The tea looper, *Ectropis grisescens* Warren (Lepidoptera: Geometridae), is a severe pest of tea plants and has caused huge economic losses in South China. The protocols described here aim to investigate, through multiple-choice bioassays, whether mature last-instar *E. grisescens* larvae can discriminate soil variables such as the substrate type and moisture content, and determine, through no-choice bioassays, the impact of the substrate type and moisture content on pupation behaviors and the emergence success of *E. grisescens.* The results would enhance the understanding of the pupation ecology of *E. grisescens* and may bring insights into soil-management tactics for suppressing *E. grisescens* populations. In addition, these bioassays can be modified to study the influences of various factors on the pupation behaviors and survivorship of soil-pupating pests.

INTRODUCTION:

Compared to the larval and adult stages of insects, the pupal stage is highly vulnerable due to the limited mobile ability of pupae, which cannot rapidly escape from dangerous situations. Pupating below the ground is a common strategy used by diverse groups of insects (*e.g.*, in the orders Diptera[1-4], Coleoptera[5], Hymenoptera[6], Thysanoptera[7], and Lepidoptera[8-12]) to protect them from above-ground predators and environmental hazards. Many of them are severe agricultural and forestry pests[1-12]. The mature larvae of these soil-pupating insects usually leave their hosts, fall on the ground, wander to find a proper site, burrow into the soil, and construct a pupal chamber for pupating[8,10].

The tea looper, *Ectropis grisescens* Warren (Lepidoptera: Geometridae), is one of the most significant defoliator pests of the tea plant *Camellia sinensis* L.[13]. Although this species was first described in 1894, it has been mistakenly identified as *Ectropis obliqua* Prout (Lepidoptera: Geometridae) in the past decades[14,15]. The differences in morphology, biology, and geographic distribution between the two sibling species have been described in some recent studies[14-16]. For example, Zhang *et al.*[15] reported that *E. oblique* mainly occurred on the borders of three provinces (Anhui, Jiangsu, and Zhejiang) of China, whereas *E. grisescens* has a much wider distribution compared to *E. oblique.* Therefore, the economic losses caused by *E. grisescens* are largely overlooked, and the knowledge of this pest needs to be extensively revised and renewed[16-19]. Our previous studies showed that *E. grisescens* prefer to pupate within soil but could also pupate when soil is not available (no-pupation-substrate conditions)[11,12].

This paper provides a step-by-step procedure to (1) determine the pupation preference of *E. grisescens* in response to factors such as substrate type and moisture content by using multiple-choice bioassays, and (2) determine the impact of abiotic factors on the pupation behaviors and emergence success of *E. grisescens* by using no-choice bioassays. All of these bioassays are

conducted under well-controlled laboratory conditions. Also, these bioassays are adapted to evaluate the influence of other factors on the pupation behaviors and survivorship of diverse soil-pupating insects.

PROTOCOL:

1. Moisture-choice Bioassays to Determine Pupation Preference of *E. grisescens*

1.1 Obtaining mature last-instar larvae of *E. grisescens*

1.1.1 Cut fresh shoots (30 - 40 cm in length) of tea plants (*Camellia sinensis* L.). Insert 25 - 30 shoots into a 250 mL triangular flask. Fill the flask with tap water. Put 3 - 4 flasks (with tea shoots) in a plastic basin (upper side: 51 cm in diameter; bottom side: 40 cm in diameter; height: 16 cm).

1.1.2 Release 1,000 - 2,000 larvae (second to fifth instar) of the laboratory colony of *E. grisescens* onto the leaves of the tea shoots in each basin. Maintain these larvae at controlled laboratory conditions [a photoperiod of 14 h of light followed by 10 h of dark (14:10 L:D), 60 - 90% relative humidity (RH), and 24 - 28 °C]. Carefully transfer the larvae onto fresh leaves by hand every 1 - 2 d. Each day remove feces and debris from the bottom of the basins.

1.1.3 Select mature last-instar larvae that fall from the leaves of the tea shoots and actively wander on the bottom of the basin. Obtain at least 240 mature larvae to ensure that enough larvae are available for the bioassays.

Note: Only select actively wandering larvae for the experiments. Do not select larvae that stay on the leaves, because these are not ready to pupate. Also, do not select prepupae with limited mobile activities because they will not actively search for the proper conditions after being released into the bioassay arenas.

1.2 Substrate Preparation

1.2.1 Collect and identify 4 types of substrate (*e.g.*, sand, sandy loam 1, sandy loam 2, and silt loam) using the hydrometer method[20]. Sterilize the soil and the sand at an 80 °C oven dryer for > 3 d, and then completely dry the soil and the sand at 50 °C for several weeks until the dry weight of the substrate samples does not change anymore over time.

1.2.2 Ground the dry soil with wooden pestles and mortars. Sift the sand and the grounded soil through a 3 mm sieve and store them in sealable plastic bags.

1.2.3 Calculate the different moisture contents of each substrate (sand, sandy loam 1, sandy loam 2, or silt loam) as follows[2]:

$$\text{Moisture content } (\%) = \frac{weight\ of\ distilled\ water}{weight\ of\ saturated\ substrate - weight\ of\ dried\ substrate} \times 100\%$$

1.2.4 Add the required amount of distilled water into the sealable plastic bags containing the dry soil or sand to prepare 5%-, 20%-, 35%-, 50%-, 65%-, and 80%-moisture substrate. Thoroughly mix the distilled water and the soil or sand.

1.3 Bioassay arena preparation

1.3.1 Equally divide the polypropylene containers (upper side: 20.0 cm in length x 13.5 cm in width, bottom side: 17.0 cm in length x 10.0 cm in width, height: 6.5 cm) into 6 chambers with waterproof polyvinyl chloride (PVC) sheets (height: 3.5 cm). Fix the PVC sheets and seal the cracks using hot glue.

Note: Completely seal any crack to prevent water permeation.

1.3.2 For each test, fill the 6 chambers using the same type of substrate with different moisture contents (5%-, 20%-, 35%-, 50%-, 65%-, and 80%-moisture) (**Figure 1a**).

[Place **Figure 1** here]

Note: Only use 1 type of substrate of different moisture contents in each test. Randomly assign the order of chambers that contain the substrate with the 6 moisture contents.

1.3.3 Paste 4 - 6 pieces of fresh tea leaves using small pieces of tape to cover the inner surface of the lids of the polypropylene containers (**Figure 1b**).

1.4 Bioassay setting and data recording

1.4.1 Release 30 mature last-instar larvae (obtained in step 1.1.3) onto the fresh tea leaves pasted on the lid of the polypropylene container. Carefully overturn the lid and tightly cover the polypropylene container.

1.4.2 Repeat each test 8x. Maintain the bioassay arenas in an environmental chamber setting at a 14:10 (L:D) photoperiod and 26 °C.

1.4.3 On day 5, count the number of pupae on the surface of the soil in each chamber. Also, dismantle the bioassays and count the number of pupae within the substrate.

Note: Only count the live pupae on or within the substrate. Check the pupae viability by observing abdominal motions after touching the pupae using forceps.

1.5 Data analyses

1.5.1 For each test, calculate the percentage of pupae found in each chamber of each replicate. Transfer the percentage data to the log ratio using the method provided by Kucera and Malmgren[21].

1.5.2 Compare the percentage of pupae (transformed data) in each chamber using a one-way analysis of variance (ANOVA). Set the significance levels at $\alpha = 0.05$ for each test.

2. Substrate-Choice Bioassays to Determine the Pupation Preference of *E. grisescens*

2.1 Repeat step 1.1 to obtain mature last-instar larvae, and step 1.2 to prepare the substrate with different moisture contents. This time, only 20%, 50%, and 80%-moisture substrate are needed.

2.2 Preparation of the bioassay arenas

2.2.1 Similar to step 1.3.1, equally divide the polypropylene containers into 4 chambers using PVC sheets. Fix the PVC sheets and seal the cracks using hot glue.

2.2.2 For each test, fill the chambers with the 4 types of substrates (sand, sandy loam 1, sandy loam 2, and silt loam) that have the same moisture content (20%, 50%, or 80% moisture) with randomly assigned orders (**Figure 1c**). Repeat step 1.3.3 to prepare the lids.

2.3. Repeat step 1.4 to set the bioassays and record the data and step 1.5 to analyze the data.

3. No-choice Bioassays to Determine the Soil-burrowing Behavior and Emergence Success of *E. grisescens*

3.1. Repeat step 1.1 to obtain the mature last-instar larvae, and step 1.2 to prepare the 4 substrates (sand, sandy loam 1, sandy loam 2, and silt loam) at 3 moisture contents (20%, 50%, and 80% moisture).

3.2. Bioassay setting

3.2.1 Add the substrate into a plastic container (upper side: 11.5 cm in diameter; bottom side: 8.5 cm in diameter; height: 6.5 cm) to a depth of 3 cm. In total, ensure that there will be 12 treatments (the combinations of 4 substrate types and 3 moisture contents). Repeat each treatment 7x.

3.2.2 Release 15 mature last-instar larvae onto the substrate of each bioassay arena. Seal the containers by tightly covering the lids. Maintain the bioassays in an environmental chamber setting at a 14:10 (L:D) photoperiod and 26 °C.

Note: There will be no need to paste fresh tea leaves on the lids as mentioned in the choice bioassays.

3.3 Data recording and analyses

3.3.1 On day 3, count the number of pupae and any dead larvae on the surface of the substrate of each replicate. Calculate the percentage of *E. grisescens* individuals that burrowed into the substrate as follows:

$$\text{Percentage of burrowed individuals (\%)} = \frac{15 - number\ of\ individuals\ on\ the\ surface}{15} \times 100\%$$

3.2.2 Record the number of emerging adults each day until no more adult emerged for 15 d. Calculate the emergence success as follows:

$$\text{Emergence success (\%)} = \frac{number\ of\ emerging\ adults}{15} \times 100\%$$

3.2.3 Compare the percentage of burrowed individuals and the emergence success among the treatments using one-way ANOVA. Set the significance levels at $\alpha = 0.05$.

REPRESENTATIVE RESULTS:

The moisture-choice bioassays showed that significantly more *E. grisescens* individuals pupated on or within the 5%- and 35%-moisture sand compared to the 80%-moisture sand (**Figure 2a**). However, significantly more individuals preferred to pupate on or within the soil (sandy loam 1 and 2 and silt loam) that had an intermediate moisture content (**Figures 2b** - **2d**).

[Place **Figure 2** here]

The substrate-choice bioassays showed that sand was significantly more preferred by *E. grisescens* individuals compared to sandy loam (1 and 2) under the 20%-moisture condition (**Figure 3a**). There was no significant difference in percentages of pupae found in the chambers containing the 4 substrates at a 50%-moisture content (**Figure 3b**). Significantly more individuals pupated on or within the sand than on or within the other substrates under the 80%-moisture condition (**Figure 3c**).

[Place **Figure 3** here]

Different moisture contents of sand did not significantly affect the percentage of burrowed individuals and emergence success of *E. grisescens* (**Figures 4a** and **4b**). Significantly fewer *E. grisescens* burrowed into dry (20% moisture) or wet (80% moisture) soil for pupating (**Figure 4a**). Also, significantly fewer adults emerged from 20%-moisture sandy loam 2 and silt loam than those that had pupated in 50%- or 80%-moisture sandy loam 2 and silt loam (**Figure 4b**).

[Place **Figure 4** here]

FIGURE LEGENDS:

Figure 1: Examples of bioassay arenas for the choice tests. (**a**) Waterproof polyvinyl chloride (PVC) sheets are used to equally divide the polypropylene containers into 6 chambers. PVC sheets are fixed with hot glue, and any cracks are carefully sealed. In this example, sandy loam 2 with different moisture contents (5%, 20%, 35%, 50%, 65%, and 80% moisture) are used to fill the chambers in the randomly assigned orders. (**b**) Fresh tea leaves are pasted on the inner side of the lids where the mature *Ectropis grisescens* larvae will be released. (**c**) PVC sheets are used to equally divide the polypropylene containers into 4 chambers, which are filled with 4 types of substrates (sand, sandy loam 1, sandy loam 2, and silt loam) at 50% moisture. This figure has been modified from Wang *et al.*[11].

Figure 2: Results from the moisture-choice bioassays. These panels show the percentages of live pupae found in each chamber containing different moisture contents (5%, 20%, 35%, 50%, 65%, and 80%-moisture) of (**a**) sand, (**b**) sandy loam 1, (**c**) sandy loam 2, or (**d**) silt loam. The data are presented as mean ± SE. The different letters indicate significant differences ($P < 0.05$). This figure has been modified from Wang *et al.*[11].

Figure 3: Results from the substrate-choice bioassays. These panels show the percentages of live pupae found in each chamber containing sand, sandy loam 1, sandy loam 2, or silt loam at (**a**) a 20%-, (**b**) a 50%-, or (**c**) an 80%-moisture content. The data are presented as mean ± SE. The different letters indicate significant differences ($P < 0.05$). This figure has been modified from Wang *et al.*[11].

Figure 4: Results from the no-choice bioassays. These panels show (**a**) the percentages of burrowed individuals and (**b**) the emergence success of *Ectropis grisescens* in response to different substrate types (sand, sandy loam 1, sandy loam 2, and silt loam) and moisture contents (20%, 50%, and 80%). The data are presented as mean ± SE. The different letters indicate significant differences ($P < 0.05$). This figure has been modified from Wang *et al.*[11].

DISCUSSION:

Pupation preferences responding to different soil variables have been studied in a few pests[6,9,22,23]. For example, to study the preference of mature larvae of *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) among different soil moisture conditions, Hulthen and Clarke[22] set a 3 x 3 Latin-square design containing 9 containers filled with soil at either 0%, 75%, or 100% field capacity, and 25 mature larvae were released onto the surface of each container. Alyokhin *et al.*[23] placed 100 containers (filled with soil) in a wooden frame, with the 36 center containers (either dry or wet) arranged in a "chessboard" pattern, and 350 - 450 late third-instar larvae of *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) were released onto the center of the 36 containers. These studies are suitable for *B. tryoni* and *B. dorsalis* larvae because most of them were recovered within the soil in the bioassay arenas[22,23]. However, these arenas were not covered. As a result, wandering larvae with a strong moving capacity may travel a long distance and escape from the arenas. Here, we provided a simple method to study the preference of soil-pupating insects regardless of their sizes and mobile abilities. Compared to previous studies, these bioassays are easy to set up. Also, multiple levels (*e.g.*, > 4) of soil variable can be studied in relatively small arenas.

It is worth noting that the data obtained from the choice tests described here cannot be directly analyzed using ANOVA because the percentage data are not independent (the sum of the percentage of pupae in each chamber always equals 1, and, therefore, the increase of the percentage of pupae in 1 chamber will cause the decrease of the percentage in the remaining ones). Here, we performed the log-ratio transformation because it is a simple procedure that "effectively removes the CSC (constant-sum constraint) from any compositional data and simultaneously retains their true covariance structure"[21]. In the present study, the survivorship of *E. grisescens* pupae was high, and we only recorded the percentage of live pupae in each chamber. However, some soil-pupating pests such as the pine processionary moth, *Thaumetopoea pityocampa* (Denis & Schiffermüller) (Lepidoptera: Thaumetopoeidae), usually exhibit a high mortality during pupating[24]. In that case, it would be proper to count both live and dead pupae.

No-choice bioassays have been widely used to investigate the effect of soil variables on the emergence success of soil-pupation pests. The substrate types and moisture contents were the most frequently studied factors in these studies[2-5,9-12]. *E. grisescens* can either pupate within or on the substrates. As a result, we recorded the percentage of burrowed individuals. This result would be important to help understand the pupation patterns of *E. grisescens*.

Both the choice and the no-choice bioassays can be modified to investigate the impact of other soil factors (such as soil density, surface compactness, content of organic matters, *etc.*) on the preference and performance of various soil-pupating insects. In a recent work, we modified these bioassays to study (1) whether the soil treated with a chemical pesticide or biocontrol agent repel pupating *E. grisescens* (both live and dead pupae were counted), and (2) the effect of such

treatments on the pupation behaviors (*e.g.*, the percentage of burrowed individuals) and emergence success of *E. grisescens*[11].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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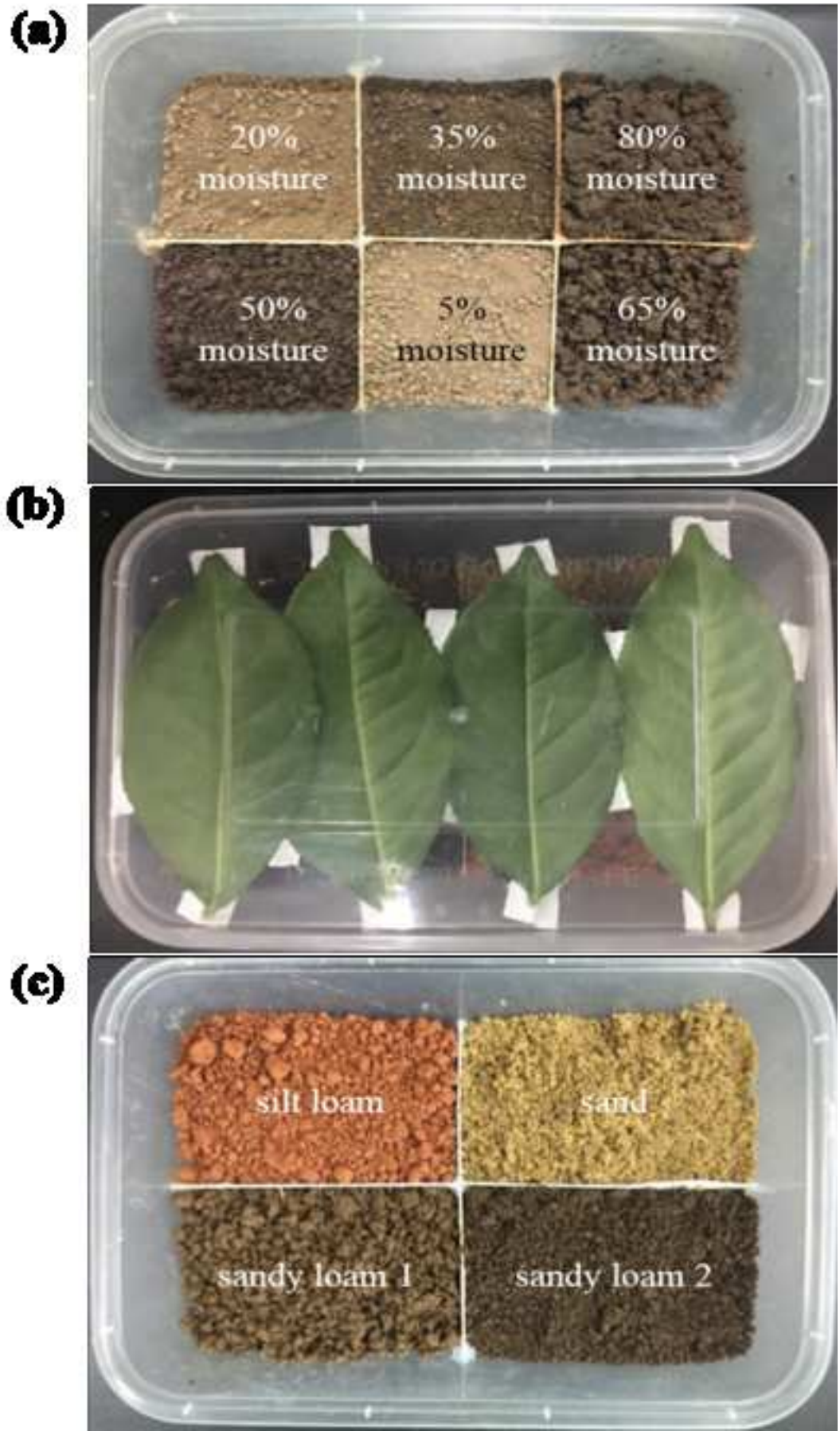

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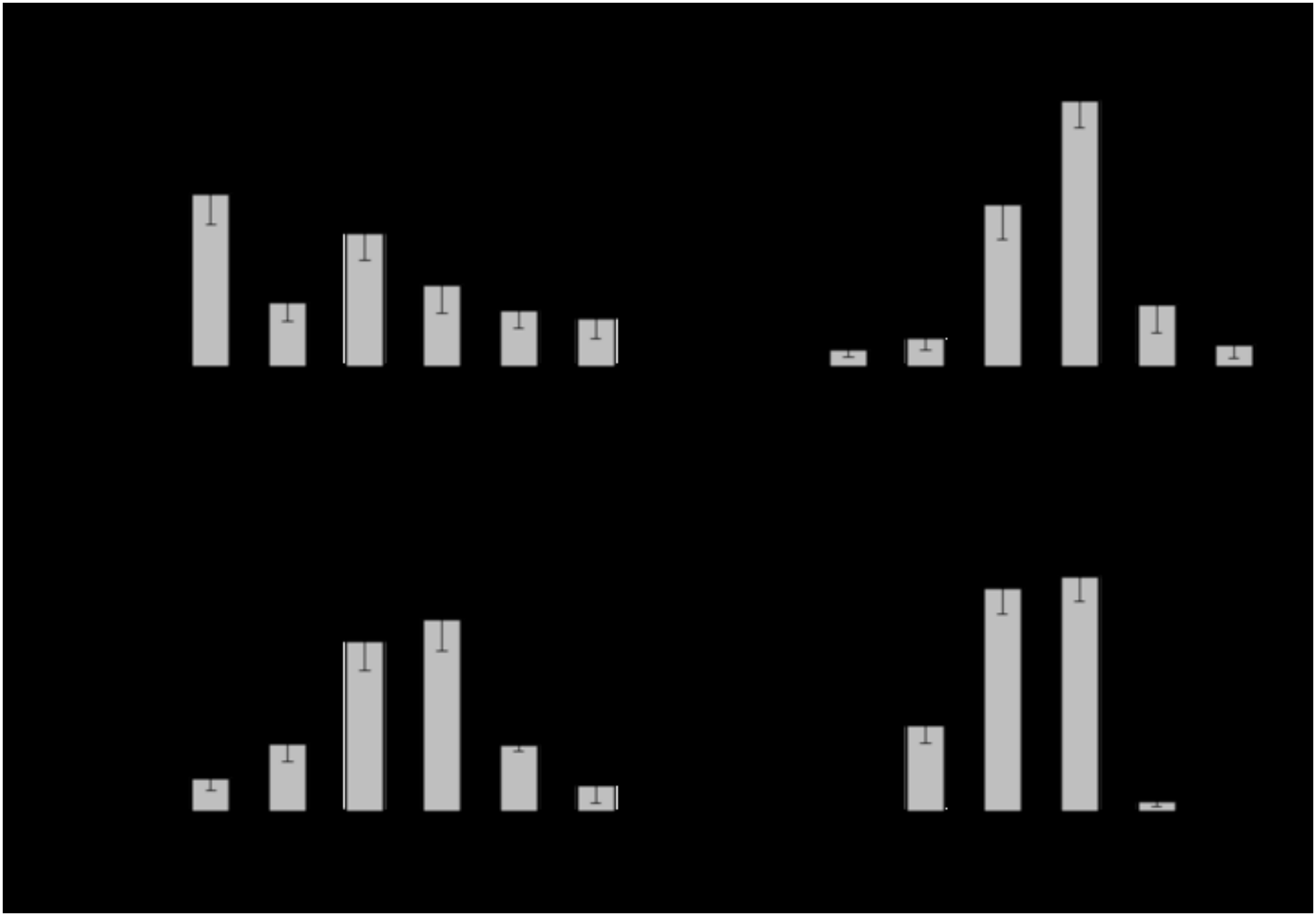

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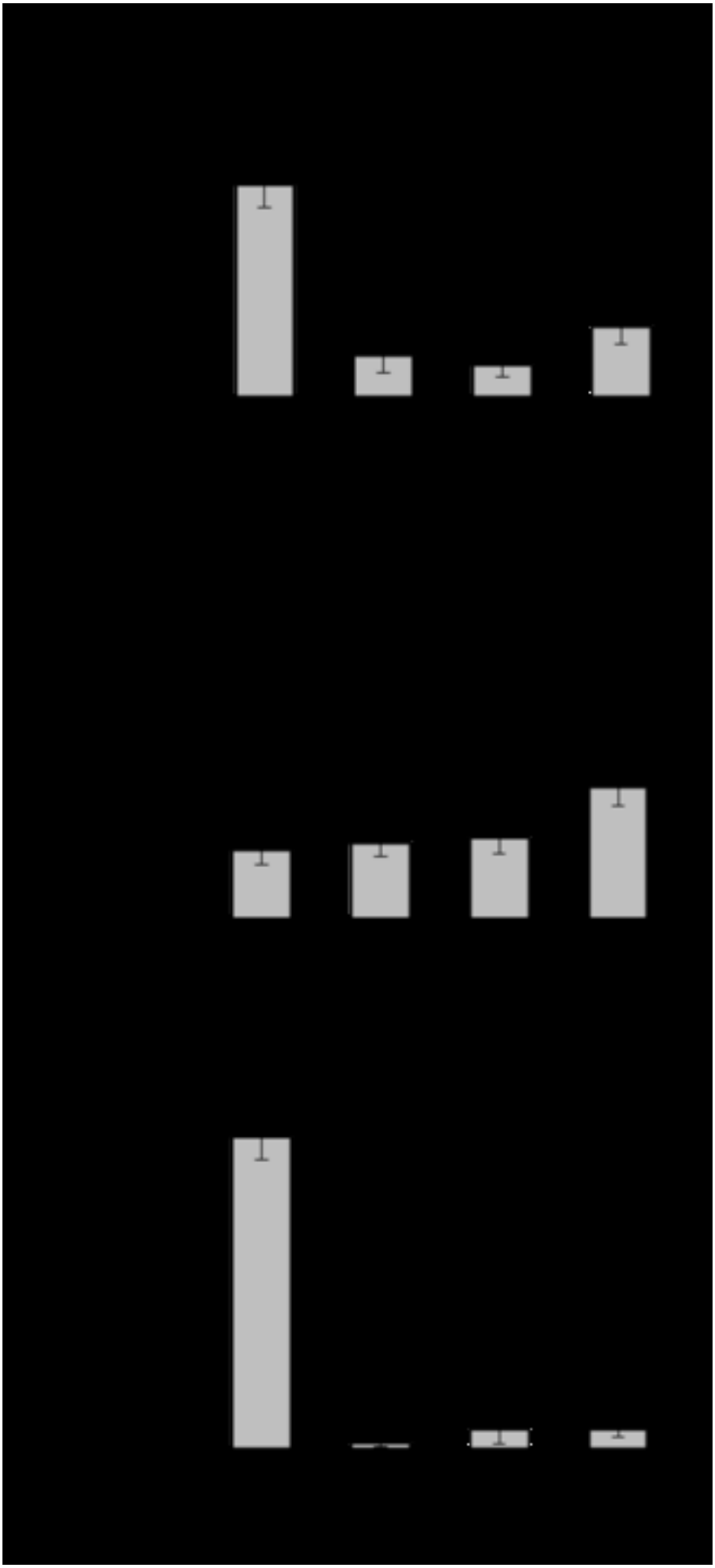

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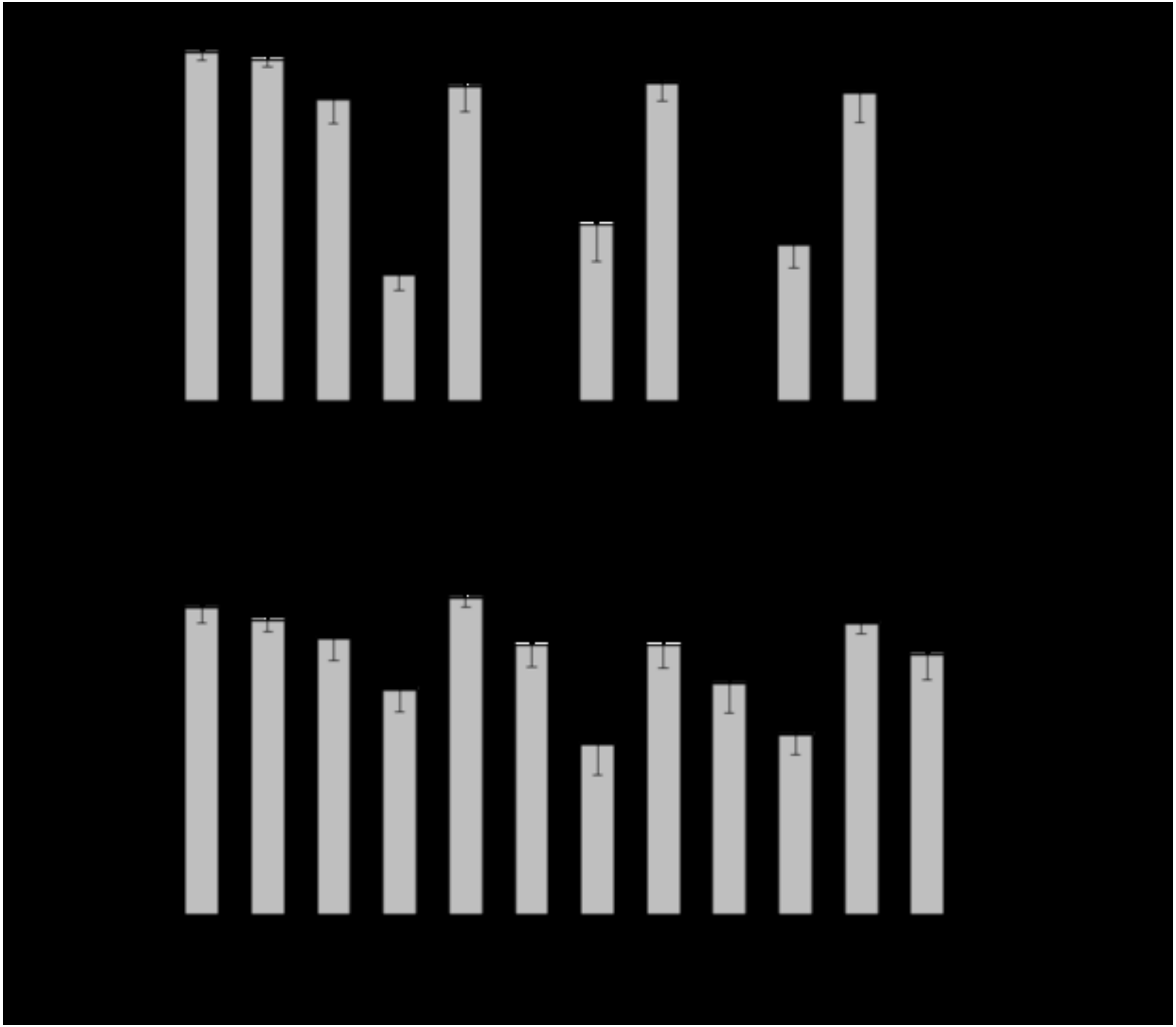

Materials Table

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Triangular flask	Bomex Chemical (Shanghai) Co., LTD	99	250 mL
Plastic basin	Chahua, Fuzhou, China	100	upper side: 51 cm in diamete
Zip lock bags	Glad, Guangzhou, China	126/133	
Polypropylene containers	Youyou Plastic Factory, Taian, China	139/155/160/161/190	upper side: 20.0 cm [L] × 13
Waterproof polyviny chloride sheet	Yidimei, Shanghai, China	141	
Tape	V-tech, Guangzhou, China	VT-710	
Oven drier	Kexi, Shanghai, China	KXH-202-3A	
Environmental chamber	Life Apparatus, Ningbo, China	PSX-280H	

er; bottom side: 40 cm in diameter; height: 16 cm

.5 cm [W], bottom side: 17.0 cm [L] × 10.0 cm [W], height: 6.5 cm

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Title of Article: Study Pupation Preference and Emergence Success of *Ectropis grisescens* by Using Choice and No-Choice Bioassays

Author(s): Cai Wang, Huifang Wang, Tao Ma, Qiang Xiao, Panrong Cao, Xuan Chen, Hongpeng Xiong, Wenquan Qin, Zhaohui Sun, Xiujun Wen

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TITLE:

Study of the Pupation Preference and Emergence Success of *Ectropis grisescens* by Using Choice and No-Choice Bioassays

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KEYWORDS:

Soil-pupation behavior, tea looper, *Ectropis grisescens*, emergence success, preference, substrate effect, substrate type, moisture content, choice test, no-choice test, *Camellia sinensis*

SHORT ABSTRACT:

Here, we present a protocol to investigate the pupation preference of mature larvae of *Ectropis grisescens* in response to soil factors (e.g. substrate type and moisture content) using choice bioassays. Also, we present a protocol of no-choice bioassays to determine factors that affect pupation behaviors and survivorship of *E. grisescens*.

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LONG ABSTRACT:

Many insects live above the ground as larvae and adults and pupate below the ground. Compared to aboveground stages of the life cycles, less attention has been paid on how environmental factors affect these insects when they pupate within the soil. The tea looper, *Ectropis grisescens* Warren (Lepidoptera: Geometridae), is a severe pest of tea plants and has caused huge economic losses in south China. Protocols shown here aim to: (1) investigate whether mature last-instar *E. grisescens* larvae can discriminate soil variables such as the substrate type and moisture content using multiple-choice bioassays; and (2) determine the impact of the substrate type and moisture content on pupation behaviors and emergence success of *E. grisescens* using no-choice bioassays. The results would enhance the understanding of pupation ecology of *E. grisescens* and may bring insights into soil-management tactics for suppressing *E. grisescens* populations. In addition, these bioassays can be modified to study the influences of various factors on pupation behaviors and survivorship of soil-pupating pests.

INTRODUCTION:

Compared to larval and adult stages of insects, the pupal stage is highly vulnerable due to the limited mobile ability of pupae which cannot rapidly escape from danger situations. Pupating below the ground is a common strategy used by some diverse groups of insects (*e.g.,*has been reported in order Diptera[1-4], Coleoptera[5], Hymenoptera[6], Thysanoptera[7], and Lepidoptera[8-12]) to protect them from aboveground predators and environmental hazards. Many of them are severe agricultural and forestry pests[1-12]. The mature larvae of these soil-pupating insects usually leave their hosts, fall on the ground, wander to find a proper site, burrow into the soil, and construct a pupal chamber for pupating[8, 10].

The tea looper, *Ectropis grisescens* Warren (Lepidoptera: Geometridae), is one of the most significant defoliator pests of tea plant, *Camellia sinensis* L.[13]. Although this species was first described in 1894, it has been mistakenly identified as *Ectropis obliqua* Prout (Lepidoptera: Geometridae) in the past decades[14, 15]. The differences in morphology, biology, and geographic distribution between the two sibling species have been described in some recent studies[14-16]. For example, Zhang et al. [15] reported that *E. oblique* mainly occurred in the borders of three provinces (Anhui, Jiangsu, and Zhejiang) of China, whereas *E. grisescens* has a much wider distribution compared to *E. oblique.* Therefore, the economic losses caused by *E. grisescens* are largely overlooked, and the knowledge of this pest need to be extensively revised and renewed[16-19]. Our previous studies showed that *E. grisescens* prefer to pupate within soil but could also pupate when the soil is not available (no-pupation-substrate conditions)[11, 12].

This paper provides a step-by-step procedure to (1) determine the pupation preference of *E. grisescens* in response to factors such as substrate type and moisture content using multiple-choice bioassays; and (2) determine the impact of abiotic factors on the pupation behaviors and emergence success of *E. grisescens* using no-choice bioassays. All of these bioassays are

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conducted under well-controlled laboratory conditions. Also, these bioassays are adapted to evaluate the influence of other factors on pupation behaviors and survivorship of diverse soil-pupating insects.

PROTOCOL:

1. Moisture-choice Bioassays to Determine Pupation Preference of *E. grisescens* in Response to Different Moisture Levels of Substrate.

1.1 Obtaining mature last-instar larvae of *E. grisescens*

1.1.1 Cut fresh shoots (30-40 cm in length) of tea plants (*Camellia sinensis* L.). Insert 25-30 shoots into a 250 mL triangular flask. Fill the flask with the tap water. Put 3-4 flasks (with tea shoots) in a plastic basin (upper side: 51 cm in diameter; bottom side: 40 cm in diameter; height: 16 cm).

1.1.2 Release 1,000-2,000 larvae (2rd to 5th instar) of the laboratory colony of *E. grisescens* onto leaves of tea shoots in each basin. Maintain these larvae at controlled laboratory conditions (14: 10 L: D photoperiod, 60-90% RH, and 24-28°C). Carefully transfer larvae onto the fresh leaves by hand every 1-2 days. Remove feces and debris on the bottom of basins each day.

1.1.3 Select mature last-instar larvae that fall from leaves of tea shoots and actively wander on the bottom of the basin. Obtain at least 240 mature larvae to ensure that enough larvae are available for the bioassays.

CAUTION: Only select actively wandering larvae for experiments. Do not select the ones that still stay on the leaves because they are not ready to pupate. Also, do not select prepupae with limited mobile activities because they will not actively search for the proper conditions after being released into the bioassay arenas.

1.2 Substrate Preparation

1.2.1 Collect and identify four types of substrate (*e.g.,* sand, sandy loam 1, sandy loam 2, and silt loam) using the hydrometer method [20]. Sterilize soil and sand at 80 °C oven dryer for > 3d, and then completely dry the soil and sand at 50 °C for several weeks until the dry weight of substrate samples will not change over time.

1.2.2 Ground dry soil with wooden pestles and mortars. Sift sand and grounded soil through a 3-mm sieve, and store in Zip lock bags.

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1.2.3 Calculate different moisture contents of each substrate (sand, sandy loam 1, sandy loam 2, or silt loam) as follows [2]:

Moisture content (%) = [weight of distilled water/ (weight of saturated substrate - weight of dried substrate)] × 100%

1.2.4 Add required amount of distilled water into Zip lock bags containing dry soil or sand to prepare 5%-, 20%-, 35%-, 50%-, 65%- and 80%-moisture substrate. Thoroughly mix distilled water and soil or sand.

1.3 Bioassay arena preparation

1.3.1 Equally divide the polypropylene containers (upper side: 20.0 cm [L] × 13.5 cm [W], bottom side: 17.0 cm [L] × 10.0 cm [W], height: 6.5 cm) into 6 chambers with waterproof polyviny chloride (PVC) sheets (height = 3.5 cm). Fix PVC sheets and seal the cracks using the hot glue.

CAUTION: Completely seal any crack to prevent water permeation.

1.3.2 For each test, fill the 6 chambers using the same type of substrate with different moisture contents (5%-, 20%-, 35%-, 50%-, 65%- and 80%-moisture) (**Figure 1a**).

[Place **Figure 1** here]

Note: Only use one type of substrate of different moisture contents in each test. Randomly assign the order of chambers that contain the substrate with the 6 moisture contents.

1.3.3 Paste 4-6 pieces of fresh tea leaves using small pieces of tape to cover the inner surface of lids of the polypropylene containers (**Figure 1b**).

1.4 Bioassay setting and data recording

1.4.1 Release 30 mature last-instar larvae (step 1.1.3) onto the fresh tea leaves pasted on the lid of the polypropylene container. Carefully overturn the lid and tightly cover the polypropylene container.

1.4.2 Repeat each test 8 times. Maintain the bioassay arenas in an environmental chamber setting at 14: 10 L: D photoperiod and 26°C.

1.4.3 On day 5, count the number of pupae on the surface of soil in each chamber. Also, dismantle the bioassays and count the number of pupae within the substrate.

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Note: Only count the live pupae on or within the substrate. Check the pupae viability by observing abdominal motions after touching the pupae using forceps.

1.5 Data analyses

1.5.1 For each test, calculate the percentage of pupae found in each chamber of each replicate. Transfer the percentage data to the log ratio using the method provided by Kucera and Malmgren[21].

1.5.2 Compare the percentage of pupae (transformed data) in each chamber using one-way analysis of variance (ANOVA). Set the significance levels at $\alpha = 0.05$ for each test.

2. Substrate-choice bioassays to determine the pupation preference of *E. grisescens* in response to different substrate types.

2.1. Repeat step 1.1 to obtain the mature last-instar larvae, and step 1.2 to prepare the substrate with different moisture contents.

2.2. Preparing of bioassay arenas

2.2.1. Similar to step 1.3.1, equally divide the polypropylene containers into 4 chambers using PVC sheets. Fix PVC sheets and seal the cracks using the hot glue.

2.2.2. For each test, fill chambers with the four types of substrates (sand, sandy loam 1, sandy loam 2, and silt loam) that have the same moisture content (20%-, 50%-, or 80%-moisture) with randomly assigned orders (**Figure 1c**). Repeat step 1.3.3 to prepare the lids.

2.3. Repeat step 1.4 to set the bioassays and record the data, and step 1.5 to analyze the data.

3. No-choice Bioassays to Determine the Soil-burrowing Behavior and Emergence Success of *E. grisescens* in Response to Different Substrate Types and Moisture Contents.

3.1. Repeat step 1.1 to obtain the mature last-instar larvae, and step 1.2 to prepare the four substrates (sand, sandy loam 1, sandy loam 2, and silt loam) at three moisture contents (20%-, 50%-, and 80%-moisture).

3.2. Bioassay setting

3.2.1 Add substrate into a plastic container (upper side: 11.5 cm in diameter; bottom side: 8.5 cm in diameter; height: 6.5 cm) to a depth of 3 cm. In total, there will be 12 treatments (the

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combinations of four substrate types and three moisture contents). Repeat each treatment 7 times.

3.2.2 Release 15 mature last-instar larvae onto the substrate of each bioassay arena. Seal the containers by tightly covering the lids. Maintain the bioassays in an environmental chamber setting at 14: 10 L: D photoperiod and 26°C.

Note: There will be no need to paste fresh tea leaf on the lids as mentioned in the choice bioassays.

3.3 Data recording and analyses

3.3.1 On day 3, count the number of pupae and any dead larvae on the surface of the substrate of each replicate. Calculate the percentage of *E. grisescens* individuals that burrowed into the substrate as follows:

Percentage of burrowed individuals (%) = [(15 – number of individuals on the surface) / 15] × 100%

3.2.2 Record the number of emerging adults each day until no more adult emerged for 15 d. Calculate the emergence success as follows:

Emergence success (%) = [number of emerging adults / 15] × 100%

3.2.3 Compare the percentage of burrowed individuals and emergence success among treatments using one-way ANOVA. Set the significance levels at $\alpha = 0.05$.

REPRESENTATIVE RESULTS:

Moisture-choice bioassays showed that significantly more *E. grisescens* individuals pupated on or within 5%- and 35%-moisture sand compared to 80%-moisture sand (**Figure 2a**). However, significantly more individuals preferred to pupate on or within the soil (sandy loam 1 and 2, and silt loam) that was at the intermediate moisture content (**Figure 2b**-**d**).

[Place **Figure 2** here]

Substrate-choice bioassays showed that sand was significantly more preferred by *E. grisescens* individuals compared to sandy loam (1 and 2) under 20%-moisture condition (**Figure 3a**). There was no significant difference in percentages of pupae found in chambers containing the four substrates at 50%-moisture content (**Figure 3b**). Significantly more individuals pupated on or within sand than other substrates under 80%-moisture condition (**Figure 3c**).

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Commented [A45]: Leave or leaf?

Commented [A46R45]: We changed “leave” to “leaf”

Commented [A47]: Why choice and no choice assay are performed on different days? Day 3 vs day 5.

Commented [A48R47]: It could be counted on day 5. But we counted the number on day 3 in the published study.

[Place **Figure 3** here]

Different moisture contents of sand did not significantly affect the percentage of burrowed individuals and emergence success of *E. grisescens* (**Figure 4a**, b). Significantly fewer *E. grisescens* burrowed into dry (20%-moisture) or wet (80%-moisture) soil for pupating (**Figure 4a**). Also, significantly fewer adults emerged from 20%-moisture sandy loam 2 and silt loam than those that at 50%- or 80%-moisture content (**Figure 4b**).

[Place **Figure 4** here]

Figure 1: Examples of bioassay arenas of the choice tests. (a) Waterproof polyviny chloride (PVC) sheets are used to equally divide the polypropylene containers into 6 chambers. PVC sheets are fixed using the hot glue, and any cracks are carefully sealed. In this example, sandy loam 2 with different moisture contents (5%-, 20%-, 35%-, 50%-, 65%- and 80%-moisture) are used to fill the chambers with randomly assigned orders. (b) Fresh tea leaves are pasted on the inner side of lids where mature *Ectropis grisescens* larvae will be released. (c) PVC sheets are used to equally divide the polypropylene containers into 4 chambers, which are filled with four types of substrates (sand, sandy loam 1, sandy loam 2, and silt loam) at 50%-moisture. This figure has been modified from Wang *et al.*[11].

Figure 2: Results from the moisture-choice bioassays. Percentages of live pupae found in each chamber containing different moisture contents (5%-, 20%-, 35%-, 50%-, 65%- and 80%-moisture) of (a) sand, (b) sandy loam 1, (c) sandy loam 2, or (d) silt loam. Data are presented as mean ± SE. The different letters indicate significant differences ($P < 0.05$). This figure has been modified from Wang *et al.*[11].

Figure 3: Results from the substrate-choice bioassays. Percentages of live pupae found in each chamber containing sand, sandy loam 1, sandy loam 2, or silt loam at (a) 20%-, (b) 50%-, or (c) 80%-moisture content. Data are presented as mean ± SE. The different letters indicate significant differences ($P < 0.05$). This figure has been modified from Wang *et al.*[11].

Figure 4: Results from the no-choice bioassays. (a) Percentages of burrowed individuals, and (b) emergence success of *Ectropis grisescens* in response to different substrate types (sand, sandy loam 1, sandy loam 2, and silt loam) and moisture contents (20%-, 50%-, and 80%-moisture). Data are presented as mean ± SE. The different letters indicate significant differences ($P < 0.05$). This figure has been modified from Wang *et al.*[11].

DISCUSSION:

The pupation preferences responding to different soil variables have been studied in a few pests[6, 9, 22, 23]. For example, to study the preference of mature larvae of *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) among different soil moisture conditions, Hulthen and

Clarke[22] set a 3×3 Latin-square design containing nine containers filled with soil at either 0, 75 or 100% field capacity, and 25 mature larvae were released onto the surface of each container. Alyokhin *et al.* [23] placed 100 containers (filled with soil) in a wooden frame, with the 36 center containers (either dry or wet) arranged in a "chessboard" pattern, and 350-450 late third instar larvae of *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) were released onto the center of 36 containers. These studies are suitable for *B. tryoni* and *B. dorsalis* larvae, because most of them were recovered within the soil in the bioassay arenas[22, 23]. However, these arenas were not covered. As a result, wandering larvae with strong moving capacity may travel a long distance and escape from the arenas. Here, we provided a simple method to study the preference of soil-pupating insects regardless of their sizes and mobile abilities. Compared to previous studies, our bioassays are easily to be set. Also, multiple levels (*e.g.,*> 4) of soil variable can be studied in relatively small arenas.

It is worth noting that the data obtained from our choice tests cannot be directly analyzed using ANOVA because the percentage data are not independent (the sum of percentage of pupae in each chamber always equals 1, and therefore the increase of percentage of pupae in one chamber will cause the decrease of percentage in the remaining ones). Here, we performed the logratio transformation because it is a simple procedure that "effectively removes the CSC (constant-sum constraint) from any compositional data and simultaneously retains their true covariance structure"[21]. In the present study, the survivorship of *E. grisescens* pupae was high, and we only recorded the percentage of live pupae in each chamber. However, some soil-pupating pests such as the pine processionary moth, *Thaumetopoea pityocampa* (Denis & Schiffermüller) (Lepidoptera: Thaumetopoeidae), usually exhibit high mortality during pupating[24]. In that case, it would be proper to count both live and dead pupae.

The no-choice bioassays have been widely used to investigate the effect of soil variables on emergence success of soil-pupation pests. The substrate types and moisture contents were the most frequently studied factors in these studies[2-5, 9-12]. *E. grisescens* can either pupate within or on the substrates. As a result, we recorded the percentage of burrowed individuals. This result would be important to help us understanding the pupation patterns of *E. grisescens*.

Both of our choice and no-choice bioassays can be modified to investigate the impact of other soil factors (such as soil density, surface compactness, content of organic matters, *etc.*) on the preference and performance of various soil-pupating insects. In a recent work, we modified these bioassays to study (1) whether the soil treated with a chemical pesticide or biocontrol agent repel pupating *E. grisescens* (both live and dead pupae are counted); and (2) the effect of such treatments on the pupation behaviors (*e.g.,*percentage of burrowed individuals) and emergence success of *E. grisescens.*

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The authors have nothing to disclose.

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