Soil Moisture Effects on Pupation Behavior, Physiology, and Morphology of *Heortia vitessoides* (Lepidoptera: Crambidae)[1]

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Abstract Previous studies show that pupating in soil is essential for *Heortia vitessoides* Moore (Lepidoptera: Crambidae) to complete its life cycle. However, little is known of the process. In the present study, we observed soil-burrowing and pupal-chamber construction by prepupae of *H. vitessoides* in 0.5-cm-wide acrylic plate interlayers. In bioassays, we also investigated pupation behaviors and pupal physiology and morphology in response to soil moistures of 5%, 25%, 45%, and 65% water saturation. Prepupae burrowed significantly deeper in soils with higher levels of soil moisture and constructed larger pupal chambers at 65% water saturation. *H. vitessoides* pupae also had significantly greater percentage biomass loss and lower body water content when the soil moistures were lower. Interestingly, pupae in 5% saturated soil were smaller and showed a significantly higher surface-to-volume ratio than at other soil moisture levels, which might pose challenges in water conservation.

Key Words *Heortia vitessoides*, moisture condition, pupa physiology, pupal chamber, soil-pupation behavior

The pupating behaviors of many insects have been investigated to reveal some commonly observed responses among diverse taxa (Alyokhin et al. 2001, Bernier et al. 2014, Chen and Shelton 2007, Renkema 2011, Wen et al. 2016, Zheng et al. 2013). For example, dry and wet soil substrates usually exert harmful effects on pupal development, indicating that soil moisture is a key factor in the pupation processes in soil (Bernier et al. 2014, Chen and Shelton 2007, Wen et al. 2016). Also, increasing the depth of pupae by adding soil or mulches to the substrate usually reduces emergence success (Chen and Shelton 2007, Renkema 2011). Such studies have provided a basis for soil management tactics (e.g., modifying moisture content, soil cultivation practices) aimed at suppressing natural populations of some agricultural and forestry pests, including *Bactrocera dorsalis* (Hendel) (Alyokhin et al. 2001, Hou et al. 2006), *Contarinia nasturtii* (Kieffer) (Chen and Shelton 2007), and *Rhagoletis mendax* Curran (Renkema 2011).

Heortia vitessoides Moore (Lepidoptera: Crambidae) is regarded as the most severe defoliating pest of *Aquilaria* plants that produce valuable agarwood (Jin et al.

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2016, Kalita et al. 2001, Qiao et al. 2013). In south China, *Aquilaria sinensis* (Loureiro) Sprenger is the preferred host plant of *H. vitessoides* (Jin et al. 2016, Qiao et al. 2013). Field surveys showed that the outbreak of *H. vitessoides* caused extensive defoliation in *A. sinensis* plantations (Jin et al. 2016, Qiao et al. 2013) and, therefore, greatly threatened of agarwood production, causing huge economic losses (Turjaman et al. 2016). Our previous studies showed that *H. vitessoides* larvae were not able to successfully pupate if they failed to dig into the soil substrate (Wen et al. 2016). In addition, choice and no-choice bioassays showed that significantly fewer *H. vitessoides* individuals successfully pupated under dry (0% water saturated) or wet (80% water saturated) soil conditions (Wen et al. 2016). Although pupation preference and emergence success were reported, gaps remain in our knowledge of the pupation processes of *H. vitessoides*.

The objectives of this study reported herein were to: (a) describe pupation behaviors using soil enclosed between layers of acrylic; and (b) define behavior, physiological, and morphological responses of *H. vitessoides* pupae to four levels of soil moisture (5%, 25%, 45%, and 65% water saturation). Pupation depth and pupal chamber sizes and shape were primary behaviors recorded, while pupal biomass and body water content and pupal size and surface-to-volume ratio were physiological and morphological factors measured in each soil moisture condition.

Materials and Methods

Insects. *Heortia vitessoides* larvae were collected from an *A. sinensis* plantation located in the Yuejingbei Experimental Station (23°9′N, 113°21′E) of South China Agricultural University, Guangzhou, China, between 5 and 30 June 2016. Fourth- or fifth-instar larvae (identified as described in Qiao et al. [2013]) were collected from *A. sinensis* trees by hand. Approximately 50–100 larvae were collected each time and transported to the laboratory within 1–2 h. These larvae were then reared in the 20-L plastic containers on a 14:10 h light:dark photoregime at room temperature (26 ± 2°C). Fresh *A. sinensis* leaves were provided daily until larvae ceased feeding and showed wandering behaviors characteristic of prepupation behavior and, thus, were ready for experimentation.

Soil preparation. Sandy loam soil (71.4% sand, 16.6% silt, and 12.0% clay, pH = 5.55, organic matter content = 7.63%, gravimetric soil water content at saturation = 53.4%) was previously collected from the field (23°09′31′′N, 113°22′07′′E) and had been used as the substrate in our laboratory colony of *H. vitessoides*. Before use, the soil was oven-dried at 80°C for >2 weeks. The dried soil was ground using a wooden mortar and pestle and sifted through a 2-mm sieve. Sifted soil (1,000 g) was stored in individual sealed Ziplock bags (25 × 30 cm, CleanWrap™, Shanghai, China). To prepare 5%, 25%, 45%, and 65% saturated soil, the required amounts of distilled water (calculated with the equation provided by Chen and Shelton [2007]) was added to the Ziplock bags containing the soil and thoroughly mixed.

Pupation behavior. A protocol provided by Zheng et al. (2011) was modified to observe pupation behaviors of *H. vitessoides* individuals in the soil. Bioassay arenas were 0.5-cm-wide interlayers made with two transparent pieces of acrylic plate (thickness = 3 mm, size = 100 × 100 mm) and three acrylic strips (thickness = 5 mm, size = 100 × 10 mm, as seen in Fig. 1). Clamps were used to hold the acrylic

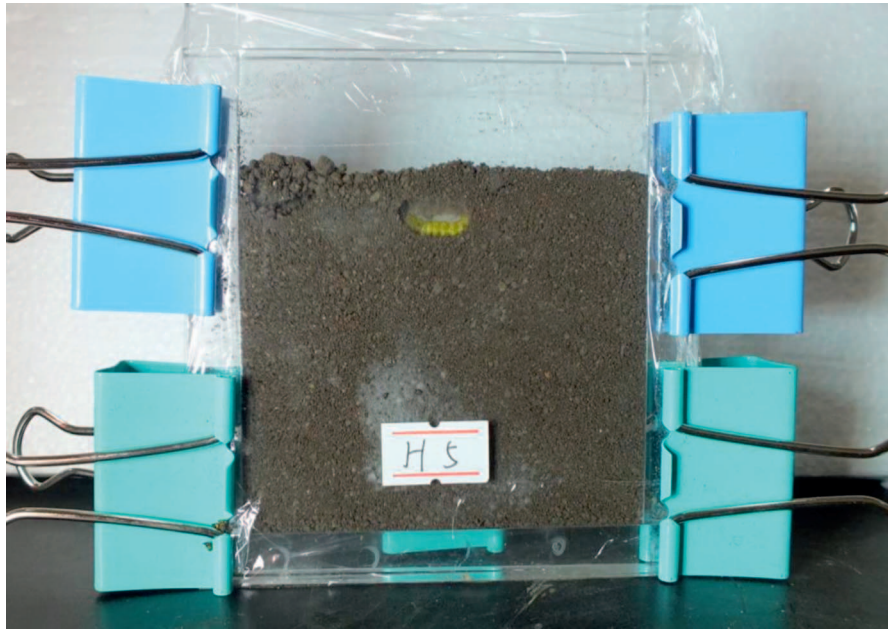

Fig. 1. The arenas used to observe burrowing behaviors of *Heortia vitessoides* larvae and formation of the pupal chamber.

plates together, keeping them upright. Sandy loam soil (45% saturated) was filled to a depth of 7 cm (Fig. 1). One last-instar wandering *H. vitessoides* larva was carefully released into the interlayer, which was then sealed with clear plastic wrap (Glad®, Guangzhou, China) and placed on a laboratory surface under room temperature (26 ± 2°C). The resultant burrowing behavior and formation of the pupal chamber were observed and recorded using a high-resolution digital camera (Fujifilm, Japan). Once construction of each pupal chamber was completed, an image was then taken daily to record transitions from larva to pupa and from pupa to adult until the adult moth emerged or the observed individual died. The arenas were maintained in an airtight plastic container with wet paper towels on the bottom to maintain high levels of humidity; that container was covered with paper to exclude light. In total, the pupation behavior of six individuals was observed during this phase of the study.

Pupation responses to soil moisture. Assay arenas were prepared using 50-ml centrifuge tubes containing 40 ml (cm^3) of the sandy loam soil (5%, 25%, 45%, or 65% saturated). Late-instar *H. vitessoides* larvae exhibiting prepupal wandering behavior were individually weighed and released into a centrifuge tube. Tubes were maintained on a 14:10 h light:dark photoregime at room temperature (26 ± 2°C).

Five days later, soil was carefully removed from each tube to expose the intact pupal chamber. The distance between the soil surface and the bottom of the pupal chamber was measured, and the chamber was removed from the tube to measure its outer and inner length and width. The pupal chambers were considered as

prolate spheroids, and the aspect ratio of the outer shell and inner space were calculated. The pupa was carefully removed from each pupal chamber, and the fresh weight, length, and width of each were measured. Each pupa was considered as a prolate spheroid, and the volume and surface area were calculated. The pupa was then examined with a stereoscope to determine the gender by using the method of Cao et al. (2013). Each pupa was then placed in a 2-ml Eppendorf tube and oven-dried (50°C) for 3 d to achieve a constant dry weight. The percentage biomass loss was calculated as % biomass loss = [(fresh larval biomass − fresh pupal biomass)/fresh larval biomass] × 100, and the body water content of each pupa was calculated as % body water content = [(fresh pupal biomass − dry pupal biomass)/fresh pupal biomass] × 100.

These data were collected from only living and healthy individuals. A range of 38–42 pupae were measured at each soil moisture level.

Data analysis. For each test, a two-way analysis of variance (PROC MIXED, SAS 9.4, SAS Institute, Cary, NC) was conducted to compare data with soil moisture as a fixed factor and gender as a random effect, followed by the Tukey's HSD test for means comparison. In all tests, significance levels were determined at $\alpha = 0.05$ level.

Results

Behavior observations. For the six insects observed, one larva failed to burrow into the soil and died at the larval stage. The other five larvae successfully constructed the pupal chambers, but only one adult successfully emerged. Those larvae dug into the soil using their heads and thoracic legs (Fig. 2A, B). Soil particles from the digging were pushed behind the burrowing larva and blocked the entrance of the tunnel (Fig. 2C). Once in the soil, the larvae constructed the pupal chamber by compressing the soil with head and legs, while at the same time spinning silk to stick the soil particles together (Fig. 2D, E). Eventually, a chamber space was successfully created, and the larva compressed any loose soil particles to the chamber wall (Fig. 2G, H), using its head to move and spread soil particles (Fig. 2I, J), and producing multiple thick layers of silks that separated soil and inner chamber spaces (Fig. 2K). After the pupal chamber was completed, the larva became shorter and turned into a prepupa and then a pupa (Fig. 2L, M). After emergence, the adult made a tunnel out of the chamber, leaving the empty pupal exuviae in the chamber (Fig. 2N–P).

Responses to soil moisture. In the tube assays, prolate spheroid-shaped pupal chambers were established in the soil in all but two of the tubes. In those two tubes, those pupae were found exposed and the pupal chambers had not been successfully completed. Pupation depth ($F = 54.73$; df = 3, 154; $P < 0.0001$) for *H. vitessoides* individuals significantly increased with increasing soil moisture (Table 1). The mean length ($F = 3.94$; df = 3, 152; $P = 0.0097$) of inner dimension of the pupal chambers constructed in 65% saturated soil moisture treatment was significantly greater than that measured in the 5% saturated soil treatment (Table 1). Moreover, the mean length ($F = 8.15$; df = 3, 152; $P < 0.0001$) and width ($F = 11.76$; df = 3, 152; $P < 0.0001$) of the outer dimensions of pupal chambers were significantly greater in the 65% saturated soil treatment than in all other soil

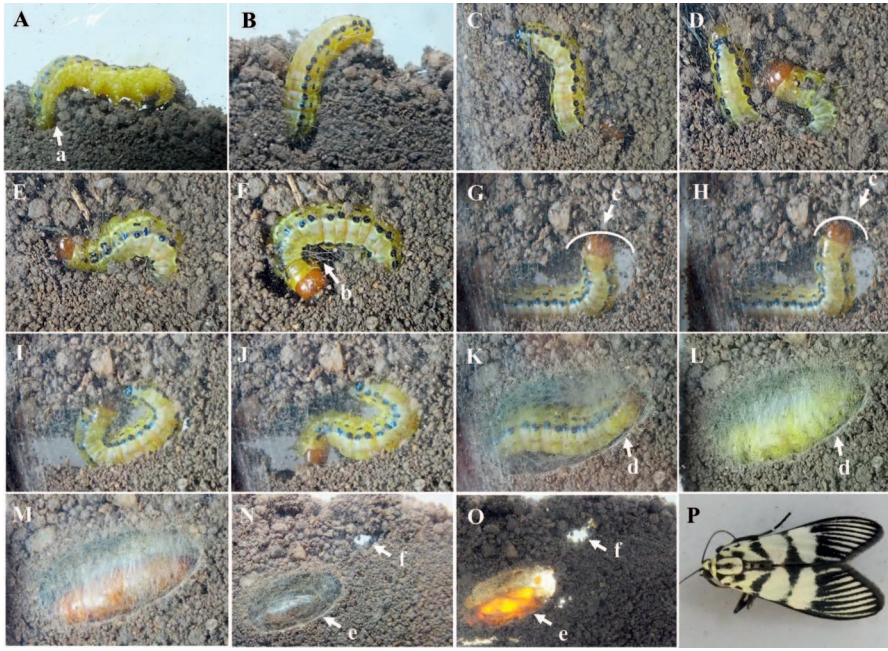

Fig. 2. Observed pupation process of *Heortia vitessoides*: (A–C) larva digging into the soil; (D–K) construction of the pupal chamber; (L–M) prepupa and pupa in the pupal chamber; (N–O) emerging tunnel; and (P) emerging adult. Arrow (a) indicates the site at which burrowing was initiated; arrow (b) shows the silk produced by the larva; arrow (c) indicates where the larva lifted up the soil to compress the sides of the chamber wall; arrow (d) indicates the multiple layers of silks that separated soil and the inner chamber space; arrow (e) shows the empty pupal exuviae remaining in the pupal chamber after the adult emergence; and arrow (f) points to part of the emergence tunnel made by the adult after emerging.

moisture treatments (Table 1). However, soil moisture did not significantly influence shape (aspect ratios) of the inner ($F = 0.42$; df = 3, 152; $P = 0.7364$) or outer ($F = 1.46$; df = 3, 152; $P = 0.2284$) surfaces of the pupal chambers (Table 1). No significant differences in fresh larval biomass ($F = 1.08$; df = 3, 153; $P = 0.3608$) and dry pupal biomass ($F = 0.51$; df = 3, 153; $P = 0.6740$) were observed among the four moisture levels; however, a significantly lower fresh pupal biomass ($F = 38.18$; df = 3, 153; $P < 0.0001$) and a significantly greater percentage biomass loss ($F = 95.90$; df = 3, 153; $P < 0.0001$) during the larval-to-pupal transition were observed in the 5% soil moisture treatment when compared with the other treatments (Table 1). Moreover, the body water content ($F = 150.60$; df = 3, 153; $P < 0.0001$) significantly increased with increasing soil moisture (Table 1). Interestingly, pupae in the 5% soil treatment had a significantly shorter pupal length ($F = 27.57$; df = 3, 154; $P < 0.0001$) and width ($F = 8.57$; df = 3, 154; $P < 0.0001$), as well as a smaller surface

Table 1. Measurements (mean ± SE) of pupation behavior and physiological and morphological traits of *Heortia vitessoides* pupae at different soil moisture levels.

Measurement	Moisture (%)*			
	5	25	45	65
Pupation behavior				
Pupation depth (mm)	9.91 ± 0.47a	13.40 ± 0.75b	18.34 ± 0.80c	24.20 ± 1.22d
Inner chamber width (mm)	5.52 ± 0.09a	5.64 ± 0.12a	5.60 ± 0.10a	5.75 ± 0.12a
Inner chamber length (mm)	16.31 ± 0.21a	16.57 ± 0.17ab	17.01 ± 0.24ab	17.39 ± 0.28b
Inner chamber aspect ratio	0.66 ± 0.01a	0.66 ± 0.01a	0.67 ± 0.01a	0.67 ± 0.01a
Outer shell width (mm)	10.99 ± 0.17a	11.08 ± 0.18a	11.12 ± 0.17a	12.18 ± 0.18b
Outer shell length (mm)	19.56 ± 0.21a	19.35 ± 0.21a	19.52 ± 0.22a	20.74 ± 0.26b
Outer shell aspect ratio	0.44 ± 0.01a	0.42 ± 0.01a	0.43 ± 0.01a	0.41 ± 0.01a
Pupa physiology				
Fresh larvae mass (mg)	144.1 ± 3.0a	142.2 ± 3.1a	146.4 ± 3.3a	140.4 ± 2.7a
Fresh pupa mass (mg)	97.9 ± 2.7a	124.5 ± 2.1b	128.2 ± 2.7b	126.3 ± 2.1b
Dry pupa mass (mg)	33.0 ± 0.9a	32.3 ± 0.8a	32.1 ± 0.9a	31.7 ± 0.7a
Percentage biomass loss (%)	32.0 ± 1.7a	14.3 ± 0.7b	12.1 ± 1.0bc	9.6 ± 1.0c
Body water content (%)	66.1 ± 0.5a	73.5 ± 0.3b	75.1 ± 0.3c	75.0 ± 0.3c

Table 1. Continued.

Measurement	Moisture (%)*			
	5	25	45	65
Pupa morphology				
Pupa width (mm)	4.09 ± 0.05a	4.27 ± 0.04b	4.34 ± 0.05b	4.39 ± 0.05b
Pupa length (mm)	13.66 ± 0.14a	14.74 ± 0.10b	14.99 ± 0.12b	14.83 ± 0.11b
Pupa surface area (mm^2)	143.01 ± 2.81a	160.68 ± 2.03b	165.95 ± 2.67b	166.40 ± 2.60b
Pupa volume (mm^3)	120.66 ± 3.70a	141.26 ± 2.87b	148.54 ± 3.81b	150.43 ± 3.88b
Surface/volume (mm^{-1})	1.20 ± 0.01a	1.14 ± 0.01b	1.13 ± 0.01b	1.12 ± 0.01b

* Means (± SE) followed by same lowercase letters within each row are not significantly different ($P < 0.05$).

area ($F = 18.57$; df = 3, 154; $P < 0.0001$) and volume ($F = 14.61$; df = 3, 154; $P < 0.0001$) and higher surface-to-volume ratio ($F = 9.92$; df = 3, 154; $P < 0.0001$) when compared with those pupae in the wetter soils (Table 1).

Discussion

Pupating behavior of other lepidopteran species has been previously described, including *Helicoverpa armigera* (Hübner) (Chen et al. 2002), *Spodoptera exigua* (Hübner) (Zheng et al. 2011), and *Manduca sexta* L. (Sprague 2013). The basic behaviors of tunneling and constructing pupal chambers are very similar and agree with our observations of these behaviors in *H. vitessoides*. However, the structure of the pupal chambers and other accessories differs. For example, *Helicoverpa armigera* constructed U- or Y-shaped entry and emergence tunnels before pupating (Chen et al. 2002), but no such emergence tunnels were observed in other species, including *H. vitessoides*. Furthermore, Sprague (2013) reported that the inner dimensions of the pupal chambers (approximately 40 cm^3) constructed by *M. sexta* were about 8 times larger than the pupae (approximately 5 cm^3). This chamber-to-pupa ratio was much higher than that observed with *H. vitessoides* in our study. The ecological and evolutionary basis for these divergent behaviors and pupal chamber structures among lepidopteran species that pupate in the soil should be further studied.

The effect of soil moisture on pupation depth has also been previously investigated with other species (Dimou et al. 2003, Hou et al. 2006, Renkema et al. 2011). Dimou et al. (2003) reported that *Bactrocera* (*Dacus*) *oleae* (Gmelin) larvae pupated deeper in soils at 50% field capacity compared with 10% field capacity. Renkema et al. (2011) also showed that when soil moisture level was high (approximately 50%) *Rhagoletis mendax* Curran preferred to pupate on the soil surface. *Ectropis oblique* Prout and *Ectropis grisescens* Warren tend to pupate on the surface of dry (5% saturation) or wet (80% saturation) soils (Y.W. and C.W. unpubl. data). Pupating on the surface may be an adaptation of these species to undesirable soil conditions. *Heortia vitessoides*, however, does not appear to be able to successfully pupate on soil surfaces and may represent a group of insects that requires soil in which to burrow to successfully pupate (Wen et al. 2016). Interestingly, *H. vitessoides* constructed larger pupal chambers in 65% saturated soil, probably because soil particles were sticky under those moisture conditions and led to a thicker chamber wall.

Sprague (2013) reported that *M. sexta* individuals lost significantly more biomass when pupated in dry rather than wet soil. Likewise, our study of *H. vitessoides* showed that the percentage biomass loss per insect increased with decreasing soil moisture. This biomass loss was largely due to decreased water content when pupation occurred in the dryer soils. Indeed, many organisms exhibit decreased surface-to-volume ratios by growing rapidly or changing morphological traits in order to reduce their vulnerability to desiccation in dry environments (Elnitsky et al. 2008, Ricci et al. 2003). In contrast, our study showed that *H. vitessoides* pupae were smaller and exhibited higher surface-to-volume ratios when they pupated in the dry (5% saturated) soil, where they may encounter higher water stress and the risk of desiccation is exacerbated (Rothermel and Luhring 2005). However, it must

be noted that these ratios are not the only factors that affect water balance. For example, epicuticular lipids played an important role in water conservation as shown in many insects (Parkash et al. 2012, Parkash and Ranga 2013). Also, the development time of the pupal stage of many insects may shorten when threatened by desiccation (Rivers et al. 2013). Future studies should be conducted to compare the composition and amount of surface lipids and the development time of *H. vitessoides* pupae under dry and wet soil conditions.

In conclusion, our study enhanced the understanding of pupation behavior and pupal physiology and morphology for *H. vitessoides*. Our results were restricted to only last-instar larvae and pupae in the small experimental arenas under laboratory conditions. It will be necessary to further study the effect of soil moisture and other factors (e.g., temperature, soil type, and compaction) on the physiology and developmental success of *H. vitessoides*. The effects of rainfall and soil moisture dynamics on *H. vitessoides* pupation and adult emergence success under natural conditions should also be investigated, developing management strategies for this insect pest.

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