N O T E

Ultrastructure of Female External Translucent Pits Useful in Sexing Gypsy Moth (Lepidoptera: Lymantriidae) Caterpillars[1]

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When rearing any insect, there are various occasions when one wishes to know the sex of an individual. In our work with gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), the question of sex determination in immature individuals has been important to know at various times. Generally, we have reared larval individuals until they pupated then easily sexing the pupae as the differentiating genital pores of the two sexes are quite easily observed, especially if viewed under some optical magnification such as a dissecting scope or hand lens. Otherwise, sexing of larvae was possible with a somewhat invasive procedure requiring a tissue sample collected from an amputated proleg (Clark 1989, *In* Wallner & McManus, U. S. Dept. Agric., Gen. Tech. Rept. NE-123) or by a staining technique of the whole early- instar larva and then squash of the caterpillar on a microscope slide (Levesque 1963, U.S. Forest Service Res. Note NE-2, 3 pp.). However, direct external morphological examination has been possible (Lavenseau 1982, Int. J. Insect Morphol. & Embryol. 11: 359-362) but has not been generally used in large part because of the need to completely restrain the living larva.

Following on statements described under "Sexing" in Winter (2000, Basic techniques for observing and studying moths and butterflies. Memoirs Lepido. Soc. No. 5, 444 pp.) in which, citing Underwood (1994, J. Lepid. Soc. 48:258-263), is the statement, "You can sex some kinds of living larvae by examining the underside of the eighth and ninth abdominal segments with a dissecting microscope" for so-called "pits". Lavenseau (1982), in examining a variety of species including gypsy moth, in general refers to these same structures "cuticular depressions" and elsewhere as "smooth and shining stria amidst the frosted cuticle." Kean and Platt (1973, J. Lepid. Soc. 27:122-129.), referring to butterflies in the genus *Limenitis* spp. (Lepidoptera: Nymphalidae), stated that females possess "longitudinally elongated translucent spots" which are absent in males. Guided by these remarks and descriptions, we

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examined gypsy moth larvae in greater detail than previously and confirm useful structures present which differentiate the sexes. Upon scanning electron microscopy (SEM) and dissecting scope examination, we settled on calling these structures "translucent pits (tp)" accepting in part the earlier terminology of earlier studies (Kean and Platt 1973, Lavenseau 1982, Underwood 1994). We developed a convenient larval restraining technique and then tested ourselves using this method to determine the reliability of sexing gypsy moth larvae.

Restraining the living caterpillar. The most critical step was to restrain the active larvae in such a way as to render it inactive and yet retaining a clear view of the ventral surface of the two posterior abdominal segments. To accomplish this, we "sandwiched" the living caterpillar between two clean standard microscope slides and exerted enough pressure, by manipulating the "sandwich" between the fingers, to constrain the larva; then invert the entire "sandwich" and hold it suspended above the stage while holding the sandwich restrained between the fingers (Fig. 1). Continual pressure was transferred to the ventral surface microscope slide and adjusted to retain the larvae directly within the visual field of the microscope. In this position, a determination of the sex of the larva was completed by examination for the presence (females) or absence (males) of translucent pits directly through the overlaying microscope slide. Larvae were viewed under a 45X stereo dissecting scope (Bausch & Lomb, Rochester, NY).

Criteria for determining larval sex. The translucent pits (tp) appear in females only approx. half way between a single seta (ss) and the ventral-lateral veruccae (vlv) on each side of the 8^{th} and 9^{th} abdominal sternites (Fig. 2A). By using this differentiating structure (tp), (compare Fig. 2A (female) and 2B (male)), we can sex individual caterpillars. Females possess a somewhat variable "pit" (Underwood 1994), "smooth and shining stria" (Lavenseau 1982), or "longitudinally elongated translucent spots" (Kean and Platt 1973), to us often resembling an eye, sometimes complete with iris, and thus our so-called translucent pits. Under the light microscope, the structure often

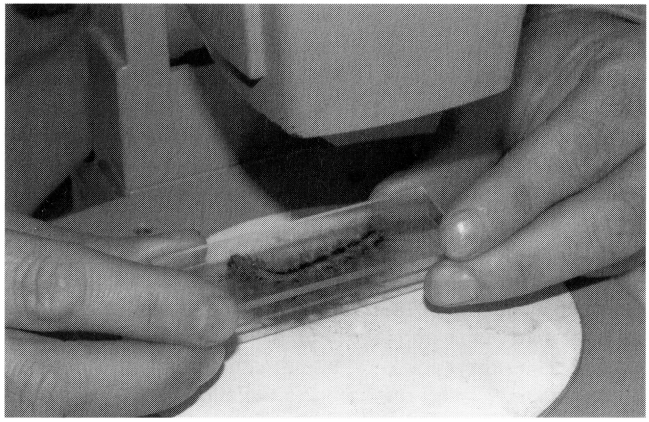

Fig. 1. Hand holding of living gypsy moth caterpillar between standard microscope slides (like a sandwich) allows viewing of female transparent pits and therefore sexing of gypsy moth caterpillars.

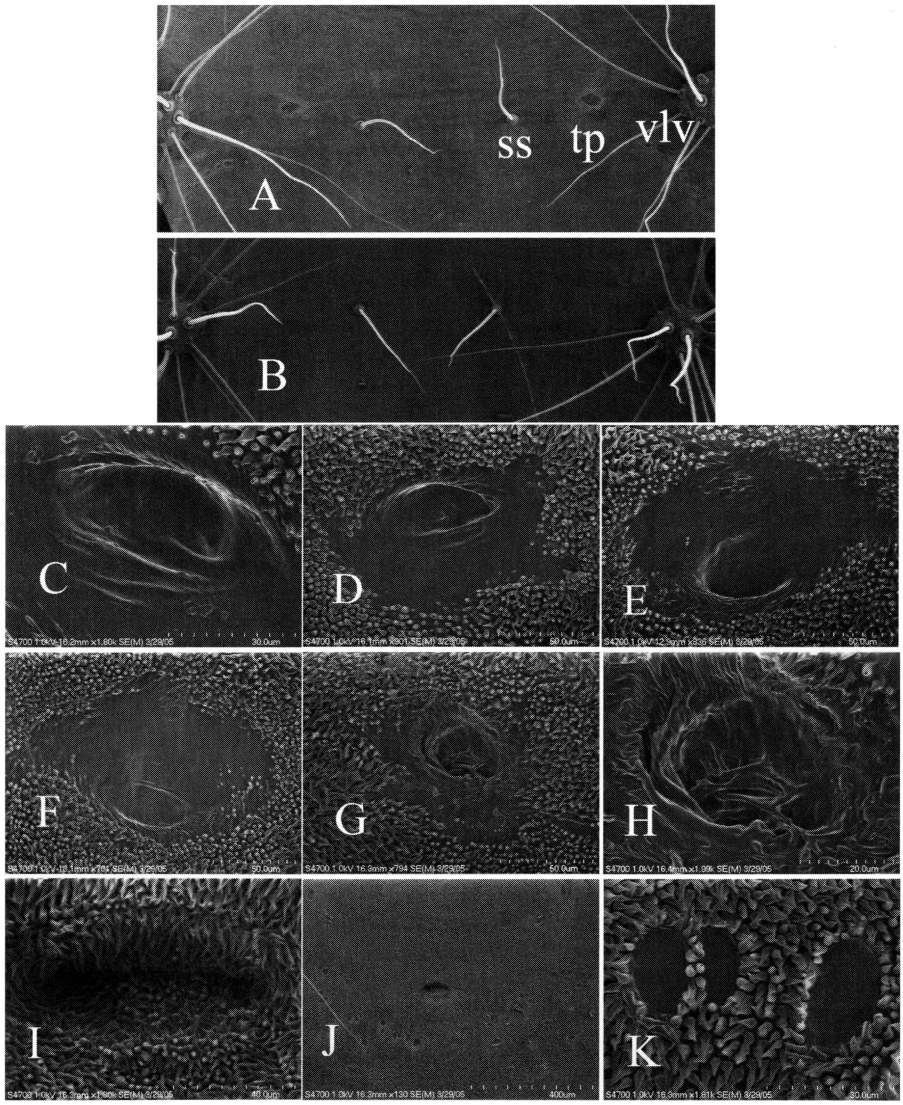

Fig. 2. Scanning electron micrographs of female (A, C thru H) and male (B, I thru K) specific characters which allow determination of sex in gypsy moth caterpillars, at least third instar and later. In micrograph (A), ss = individual seta, tp = transparent pit, and vlv = ventral lateral veruccae, which possess from about 10-15 long setae. (C - H) illustrate the variation in the female transparent pit some suggestive of an external opening (esp. G & H, an enlargement of G). Image (I) is the centrally located male genital pore surrounded by a partial halo of reflective spots (J), likely apodemes, which are shown in detail (K).

was associated with discoloration from the general color of the integument at the site of these translucent pits. Males lack translucent pits at the same site and the integument looks uniform (Fig. 2B). Males possess a somewhat pronounced semicircle of light-reflective spots medial on the anterior edge of the 9th sternite and central within this semicircle can often be seen a pit, or slight invagination (Fig. 2I), which sometimes shows a darker coloration than the surrounding integument (Fig. 2J). Reliance on color difference, however, is not dependable.

Test of the sexing technique. Each of us tested our technique by determining the sex of a series of larvae, isolating groups of larvae into paper rearing cups on the basis of our determinations, and then rearing these larvae until pupation. Upon pupation, sex of the pupae was determined as a confirmation of the larval sexing technique. Records of the number of properly determined larvae were made as pupae were removed from the rearing containers.

SEM imaging. To investigate in detail the ultrastructure of these translucent pits, we removed the ventral integument of the posterior three abdominal segments of frozen larvae, stretched and pinned these small "hides" over paper to prevent shrinkage, and held them for air drying before these segments were examined by field emission scanning electron microscopy (Hitachi S-4700, Delaware Biotechnology Center, University of Delaware, Newark).

In our normal sexing process, we found that the mere presence of translucent pits will readily distinguish "female" from male larvae but we found it best to view two or more locations before accepting a determination of "male". Some of the individual translucent pits, being quite variable, were not always easily distinguished but collectively a composite view made determination both possible and more reliable.

In our trial testing our sexing technique, all females (n = 52), third to sixth instars, were accurately sexed while all but one male among the total (n = 52) males was accurately sexed for an overall 99.0% successful sex determination. We found the technique to be highly reliable, however, it appears more difficult and therefore less reliable using the younger (3^{rd}) instars. Using late stage larvae, the sexing technique for gypsy moth proved to be completely dependable. The most difficult aspect of the entire process, was positioning and holding the squiggling upside-down larval "sandwiched" on the microscope slide stage. Once the handling technique was mastered, it became possible to sex approximately 3 larvae per minute. Once the clear view of the posterior sternites could be obtained, larval sex determination could be determined quite rapidly.

The scanning electron micrographs clearly revealed variability within the individual structures of the tp both on the same individual and between different specimens (Fig. 2C thru 2H). The collective appearance of tp suggest eyes (Fig. 2C-E), and/or similarities to a clear patch or lake appearance (Fig. 2F). Some tp's clearly suggest a morphological internal opening (Fig. 2E and 2G) and that such an opening is protected by a matrix of buttresses or supports (Fig. 2H) for supportive strength while designed to retain a clear and free open canal. If such an opening indeed exists, it immediately poses the question of what is the possible function. Lavenseau (1982) states that these are the "ectodermal infoldings forming the genital imaginal disks" and that eventually "these give rise to the terminal part of the genital duct: bursa copulatrix, spermatheca, vagina, and accessory gland." Could they possibly also possess an opening? If so, then any function clearly remains a question for further investigation.

Males lack the tp's but do possess a pit or apparent invagination (Fig. 2I) on the

midline of the anterior part of the 9th abdominal sternite. In a light microscope view, this pit sometimes appears as a discoloration which is central to a rough arc of light reflective spots (Fig. 2J) which may be the external appearance of internal apodemes (Fig. 2K). This latter illustration also well illustrates the ultrastructure of the overall ventral integument.

This larval sexing technique may well aid in many different situations where determination of the sex of individual gypsy moth caterpillars is desirable.

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