were supplied by the Illinois Natural History Survey, the Illinois Agricultural Experiment Station, and the Office of International Agriculture, College of Agriculture, University of Illinois, Urbana.

LITERATURE CITED

BARNES, W. 1904. New species of North American Lepidoptera. Can. Entomol. 36: 165–173.

GODFREY, G. L. 1972. A review and reclassification of larvae of the subfamily Hadeninae (Lepidoptera, Noctuidae) of America north of Mexico. U.S. Dept. Agr. Tech. Bull. 1450. 265 p.

METHODS FOR EXTERNALLY SEXING MATURE LARVAE AND PUPAE OF *LIMENITIS* (NYMPHALIDAE)

PHILIP J. KEAN AND AUSTIN P. PLATT

Department of Biological Sciences, University of Maryland Baltimore County, Catonsville, Maryland 21228

Little published information exists regarding accurate methods for sexing the larvae and pupae of butterflies. It is well-known, however, that larger larvae and pupae within a brood generally develop into females, whereas, the smaller ones usually turn out to be males. In addition, the majority of males often will eclose at the beginning of a brood, whereas, the latter portion of the brood will consist almost exclusively of females. Nevertheless, numerous exceptions occur, and such methods cannot be considered to be very accurate.

Among the larger moths (Saturnidae and Sphingidae), morphological differences such as the relative breadth of the pupal antennae and subtle differences in the ventral genital plates of pupae have been used for predicting the sex of the imago (Villiard, 1969), and methods for sexing mature larvae of the tobacco hornworm (*Manduca sexta* Johanssen) are known (W. Bowers, personal communication). Recently, other methods have been reported for sexing both the larvae and pupae of the codling moth, *Laspeyresia pomonella* L. (MacLellan, 1972).

During laboratory hybridization studies on the Nearctic *Limenitis*, which were initiated in 1966 and are still in progress (Platt, 1969; Platt, Frearson & Graves, 1970; Platt & Greenfield, 1971), it became apparent that in inter-specific hybrid crosses, either excessive or complete heterogametic (female) inviability often occurs (Haldane, 1922; Remington,

1958). Since egg hatching within hybrid broods sometimes exceeds 50%, it was at once apparent that some female hybrid larvae were initially viable, but then died at certain stages during development. The need for positively determining the sexes of dying hybrid larvae and malformed pupae prompted this study.

The purposes of this paper are to report means for positively sexing the mature larvae and pupae of North American *Limenitis*, and to point out how these and similar methods can add a new dimension to studies of lepidopteran genetics.

MATERIALS AND METHODS

Larvae of *L. archippus* Cramer and *L. arthemis* Drury were used principally in these studies. The former were collected as over-wintering third instar larvae found in hibernacula during January and February, 1972, and represent stocks collected from Colchester (Chittenden Co.) and Starksboro (Addison Co.), Vermont, as well as a Maryland stock collected from the University of Maryland Baltimore County (Catonsville) campus. The latter species is represented by two broods of over-wintering third instar larvae, which represent F_1 crosses obtained from wild-caught *L. arthemis–astyanax* females collected from the intergrading population at Shutesbury (Franklin Co.), Massachusetts (see Platt & Brower, 1968), and by a third inbred *L. a. astyanax* Fabricius stock obtained several years ago from the Baltimore, Maryland vicinity.

All hibernating larvae were kept in total darkness in a refrigerator within their hibernacula under moist conditions for a minimum of several weeks prior to study. Each hibernaculum then was individually isolated in a labeled plastic-covered styrofoam cup containing leaves of foodplant (*Salix babylonica* L. for *L. archippus* larvae, and *Prunus serotina* Ehrh. for *L. arthemis–astyanax* larvae). The cups were kept in covered transparent shoeboxes containing moist paper towels. The larvae were reared at room temperature in a closed photoperiod chamber under 20 hrs. of fluorescent illumination per 24 hr. day. Mature larvae were permitted to pupate on sticks placed across the tops of the cups.

As each larva emerged from its hibernaculum, it was assigned an individual number. The next day each was sexed independently, using external markings (Fig. 1), which will be described in detail in the results section of this paper. A Wild 3M stereoscope with a Pentax camera back was used for examining and photographing the larvae and pupae. The day following each subsequent larval moult (to the fourth and fifth instars), each larva was again sexed independently, without reference to the previous sexing information. In each instance, the presence or

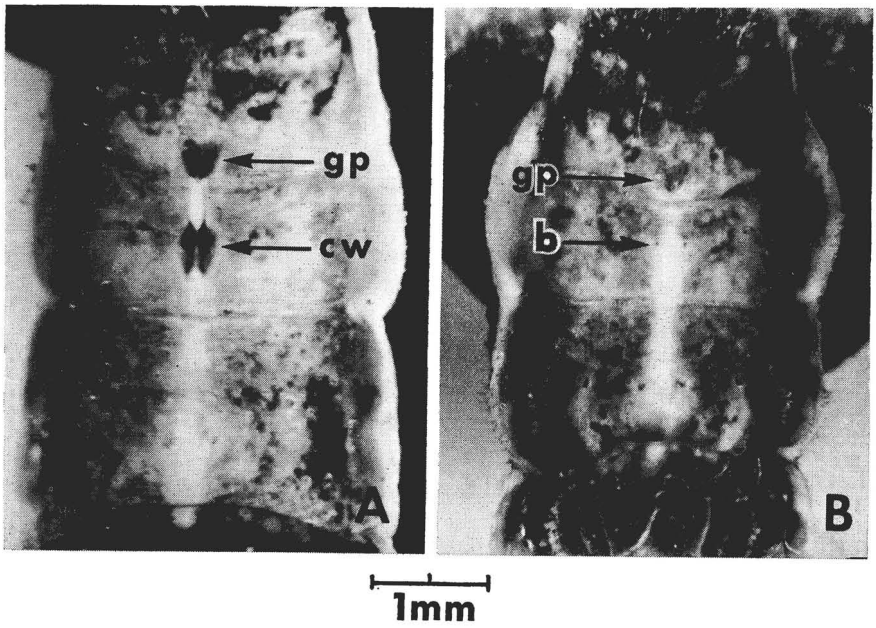

Fig. 1. Morphological sex differences on the ventral surface of the eighth abdominal segment of female (A) and male (B) *Limenitis* larvae. The larvae are oriented with their posterior regions toward the top of the photographs. gp = genital pore; cw = chitin windows of female larva; b = pair of single bristles of male larva.

absence of the same external markings was used to determine the larval sex. Upon metamorphosis, both pupae and adults likewise were sexed independently, using different morphological characters. Fig. 2 shows the morphological traits used for sexing *Limenitis* pupae. All larvae and pupae which died during development were deleted from the data, because the sex of the imago could not be positively determined.

RESULTS

External Sex Characters of Larvae. Third through fifth instar female larvae always possess two small transparent cuticular patches, which we have termed "chitin windows." They are located on the mid-ventral surface of the eighth abdominal segment (Fig. 1A). These transparent spots appear to be dark against the grey-white mid-line, because the green gut of the larva shows through from beneath them. These spots, or chitin windows, lie anterior to a similar bifurcate dark crescentic patch (Figs. 1A, B) located just behind the anterior edge, in the middle of the ventral region of the ninth abdominal segment. The latter spot is

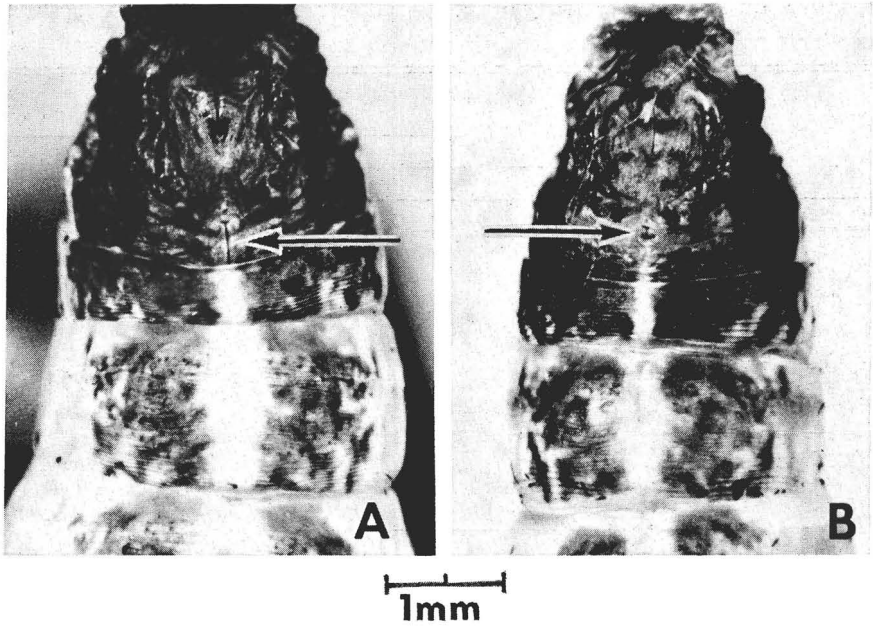

Fig. 2. Morphological sex differences on the ventral genital plates of the eighth and ninth abdominal segments of female (A) and male (B) *Limenitis* pupae. The differences described in the text are indicated by the arrows.

present in larvae of both sexes and apparently marks the primordial genital region. The functions and adult structures (if any) arising from the chitin windows presently are not known, although the female genital structures form in this region of the pupa. Male larvae (Fig. 1B) lack the chitin windows, and so appear to possess an unbroken white mid-line on the ventrum of the eighth abdominal segment. This difference can be determined readily with the naked eye in mature larvae. However, it is best to check all larvae with a stereo-microscope, using low to medium magnification. When this is done, two very small dark bumps, each containing a single short curved bristle, can be seen on the white ventral mid-line of the male larva's eighth abdominal segment. The position of these bumps is comparable to that of the chitin windows of female larvae.

External Sex Characters of Pupae. Limenitis pupae, likewise, can be sexed externally by examination of the morphology of the genital plates located ventrally, and immediately anterior to the base of the lateral processes of the cremaster. These plates cover the mid-ventral region of the entire eighth, and the anterior margin of the ninth, abdominal segments. Microscopic examination is necessary to observe the male and

TABLE 1. Results of externally sexing *Limenitis* larvae and pupae by independent examination at each stage.

A. Correct sexing observations (percent accuracy given in parentheses):

Species	Numbers ♂♂	Numbers ♀♀	Numbers Total	Developmental Stages: Larval instars 3rd	Larval instars 4th	Larval instars 5th	Pupae	Adults
L. archippus	32	26	58	41 (71%)	55 (95%)	58 (100%)	58 (100%)	58 (100%)
L. arthemis–astyanax[1]	10	11	21	13 (62%)	16 (76%)	21 (100%)	21 (100%)	21 (100%)
Totals	42	37	79	54 (68%)	71 (90%)	79 (100%)	79 (100%)	79 (100%)

B. Inaccurate sexing observations, given by sex of adult individual (based on a total of 237 observations:

Species	Larval Instars: 3rd ♂♂	3rd ♀♀	3rd sub totals	4th ♂♂	4th ♀♀	4th sub totals	Grand Totals
L. archippus	6	11	17	2	1	3	20
L. arthemis–astyanax[1]	4	4	8	3	2	5	13
Column Totals	10	15	25	5	3	8	33

[1] Includes 3 *L. arthemis arthemis* Drury, 14 *L. arthemis prosperpina* Edwards, and 4 *L. arthemis astyanax* Fabricius.

female differences clearly. Female pupae possess a longitudinally slotted mid-ventral pad (Fig. 2A). The slot always extends forward across the eighth segment (where the larval chitin windows were located) to the posterior margin of the seventh abdominal segment, appearing to arise from the segmental fold, at right angles to it. This slotted pad seems to be homologous with the distal ends of the female genital ducts in the imago. Male pupae (Fig. 2B) lack this vertically slotted pad, but possess, instead, two small paired swellings, located posterior to a prominent horizontal fold, which forms the base of a medial flattened isoceles triangular plate. This plate is circumscribed by two anterolateral oval plates. This entire small crescentic structure is restricted to the ninth abdominal segment, leaving the mid-ventral region of the eighth abdominal segment intact.

Accuracy of the Sexing Methods. The validity of using the external morphological features described above to sex the larvae has been verified in two ways. First, a small number of larvae of both sexes were

dissected, and the developing gonads identified. (In mature larvae of Lepidoptera, the gonads lie beneath the body wall in the dorsal region of the fifth abdominal segment. In *Limenitis*, this region is the central area of the grey-white larval saddlepatch. The developing ovaries of female larvae are paired flattened whitish strands of delicate wavy tissue, often imbedded in fat. The paired reddish-brown oval male testes are somewhat easier to locate in larvae; the latter fuse into a single medial rounded testis in the adult butterfly.) Secondly, 79 larvae of *L. archippus* and *L. arthemis–astyanax* were independently sexed during the third, fourth, and fifth larval instars, and later both as pupae and as adults (Table 1A). External sexing of mature larvae and of pupae was accomplished with an accuracy of 100%. Third and fourth instar larvae were sexed with 68% and 90% accuracy, respectively. Only a total of 33 (14%) inaccurate sexing observations were made in 237 independent larval observations. The majority of erroneous sexings occurred when examining third instar larvae (Table 1B). In most cases, this happened because the chitin windows (which are present) were not clearly visible in the small female larvae.

Discussion and Conclusions

These methods for externally sexing the fifth instar larvae and the pupae of North American *Limenitis* are 100% reliable. Fourth instar larvae have been sexed with an over-all accuracy of 90%. The methods may be somewhat more reliable for *L. archippus* larvae than for the larvae of *L. arthemis* at this instar (Table 1A). Because of their smaller size (approximately 12 mm long by 3 mm wide, or less), third instar larvae are much more difficult to sex. Our over-all accuracy is only 68% for this stage, with the sexing observations on *L. archippus* larvae again being more reliable than those carried out on larvae of *L. arthemis–astyanax*. We conclude that our methods cannot be considered completely reliable for the third instar. Because of the small size and delicateness of younger larvae, attempts were not made to sex them in the first and second instars.

These larval and pupal sexing methods have been tried on a few larvae of the Western species of Nearctic *Limenitis* (*L. lorquini* Boisduval and *L. weidemeyerii* Edwards). The immature stages of both of these species also can be sexed accurately using these characters. In fact, it is probable that species in closely related genera, such as *Adelpha, Apatura, Neptis, Parthenos,* etc., and, perhaps even more remotely related nymphalines, can be accurately sexed in their immature stages using either these, or similar morphological criteria.

Since sex is a genetically determined trait, the ability to sex larvae and pupae of certain Lepidoptera externally, provides a means of establishing genetic ratios prior to the adult stage. As has been implied earlier in this paper, this becomes a most useful tool in cases involving inter-specific hybridization.

For example, accurate sex ratios now can be established for crosses in which heterogametic inviability occurs during larval growth. Among the species of Nearctic *Limenitis*, inter-specific hybridization occurs only rarely in nature, but can be accomplished readily in the lab by hand-pairing the insects (Remington, 1958; Platt, 1969; Platt & Greenfield, 1971). Although the number of larvae hatching from eggs of such crosses sometimes exceeds 50%, a number of the developing larvae often die prior to, or during pupation. Are these, perhaps, mostly females, since only males are found to emerge from normally formed pupae (especially in hybrid crosses involving the broadly sympatric species, *L. archippus*)?

Preliminary evidence suggests that the answer to this question is *yes*: Recently, 21 hybrid larvae, representing three broods of *L. arthemis–astyanax* × *L. archippus* crosses have produced malformed pupae at the end of the fourth instar. Each pupa has been sexed as female externally, and no definite males have been found among them, although it is possible that some of the most malformed ones may represent inter-sex pupae. Several other hybrid larvae have grown slowly to immense size in the fifth instar, but then have failed to metamorphose. Each of these, likewise, has been externally sexed as a female.

Therefore, our methods of externally sexing the immature stages of *Limenitis* provide a means of confirming the developmental stages during which female inviability occurs. Within hybrid female larvae, species incompatibilities in juvenile hormone and ecdysone are implicated, but must be substantiated by further work. Using the external sexing methods to identify female hybrid larvae prior to death, it may be possible to treat them with the appropriate synthetic insect hormones, in order to enable them to pass through a normal metamorphosis. Such techniques may prove exceedingly useful in furthering our present knowledge of evolution among related species of Lepidoptera.

SUMMARY

Methods for accurately sexing the fourth and fifth instar larvae and the pupae of the North American species of *Limenitis* by means of external morphological differences are reported. Female larvae possess a pair of prominent dark spots (termed chitin windows) on the ventral white mid-line, located centrally on the eighth abdominal segment; male

larvae lack these spots. Female pupae possess an antero-posterior slotted pad on the ventrum of the eighth abdominal segment, whereas male pupae possess a smaller genital pore, consisting of two bulbous swellings lying just posterior to a prominently horizontal fold, the entire structure being restricted to the ninth abdominal segment. The eighth abdominal segment of male pupae possesses no mid-ventral pad with an antero-posterior slit, as it does in female pupae. These methods proved 100% effective for sexing mature larvae and pupae. Fourth instar larvae were sexed with an accuracy of 90%. However, small third instar larvae could be sexed only with an accuracy of 68%. The genetic implications of being able to externally sex immature stages of *Limenitis*, with respect to interspecific hybridization studies, are discussed briefly.

ACKNOWLEDGMENTS

We are greatly indebted to F. M. Mills, III of the University of Maryland Baltimore County for his initial studies on this topic, and to Pamela C. Platt for assistance in collecting the larval stocks. We wish to thank Dr. William Bowers, Department of Entomology, of Cornell University at Geneva, New York, for providing the information regarding sexing procedures of certain sphingid larvae. Dr. L. P. Brower of Amherst College and Dr. T. D. Sargent of the University of Massachusetts at Amherst provided helpful comments regarding certain portions of this manuscript.

LITERATURE CITED

HALDANE, J. B. H. 1922. Sex-ratio and unisexual fertility in hybrid animals. J. Genet. 12: 101–109.

MACLELLAN, C. R. 1972. Sex ratio in three stages of field collected codling moth. Can. Entomol. 104: 1661–1664.

PLATT, A. P. 1969. A simple technique for hand-pairing *Limenitis* butterflies. (Nymphalidae). J. Lepid. Soc. 23: 109–112.

———. & L. P. BROWER. 1968. Mimetic versus disruptive coloration in intergrading populations of *Limenitis arthemis* and *astyanax* butterflies. Evolution 22: 699–718.

———, S. D. FREARSON & P. N. GRAVES. 1970. Statistical comparisons of valval structure within and between populations of North American *Limenitis* (Nymphalidae). Can. Entomol. 102: 513–533.

———. & J. C. GREENFIELD, JR. 1971. Inter-specific hybridization between *Limenitis arthemis astyanax* and *L. archippus* (Nymphalidae). J. Lepid. Soc. 25: 278–284.

REMINGTON, C. L. 1958. Genetics of populations of Lepidoptera. Proc. 10th Intern. Congr. Entomol. 2: 787–805.

VILLIARD, P. 1969. Moths and How to Rear Them. Funk & Wagnells, New York. p. 242.