

## METHODS FOR SEXING LEPIDOPTERA LARVAE USING EXTERNAL MORPHOLOGY

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**Abstract.** This paper describes a method for determining the sex of larval Lepidoptera using external morphology. This method, previously developed by others but published in journals with limited distribution, uses sex-specific pits located ventrally on the 8th and 9th abdominal segments. This paper also describes a new technique for clearing and staining newly hatched larvae so that these sex-specific pits can be identified readily. Finally, this paper extends the applicability of this method to four other species in three butterfly families (Papilionidae, Nymphalidae, and Pieridae).

**Additional key words:** sex determination, larval morphology.

The ability to sex live Lepidoptera larvae would allow for novel experiments on sex-specific differences in behavior, physiology, and development, as well as hormonal and metabolite assays, to name a few. Determination of sex ratio and sex-specific mortality typically has been limited to those larval stages where sex can be determined by dissection. Yet in most species gonad differentiation does not occur until at least the third instar, and in some species such as *Papilio zelicaon* Lucas (Papilionidae), even last instar larvae are impossible to sex by examining the gonads. Moreover, sexing by dissection necessarily involves sacrificing the animals—thereby precluding any further study of these individuals.

This paper describes a method of wide application for sexing Lepidoptera larvae using external morphology. It was developed by others but published in journals with limited distribution (Joseph & Karnavar 1991, Lavenseau 1982, Muraleedharan & Muraleedharan 1989, Stehr & Cook 1968) and seems to be little known or used. Aside from *Pieris rapae* L. (Pieridae), this method has been tested previously only in the moth families Psychidae, Pyralidae, Notodontidae, Lymantridae, Noctuidae, Arctiidae, and Attacidae (Stehr & Cook 1968, Lavenseau 1982, Muraleedharan & Muraleedharan 1989, Joseph & Karnavar 1991).

Larvae can be sexed live once they are large enough, typically at least 5 mm in length, to allow one to clearly see the last four abdominal segments under a dissecting microscope. All instars of preserved larvae can be sexed. This paper extends the applicability of this method to four other species within three butterfly families. It also presents a new technique for clearing and staining very young preserved larvae, including first instars, so that this method can be used to determine their sex.

## MATERIALS AND METHODS

Fifth instar larvae of *Eucheira socialis* Westwood (Pieridae), *Battus philenor* Linnaeus (Papilionidae), *Junonia coenia* Hübner (Nymphalidae), and *Papilio zelicaon* were sexed readily by examining the 8th and 9th abdominal segments using a Wild dissecting microscope. I first sexed individuals using external morphology, then dissected and sexed the same individuals by examining their gonads. I examined at least 10 larvae, five of each sex, from each of the four species of butterflies. All sex determinations were confirmed by dissection for all species except *Papilio zelicaon* where gonadal differentiation was insufficient in fifth instar larvae to determine sex. For this species, I sexed individuals by external morphology, then reared them out and sexed them as adults.

First instar larvae of *E. socialis* preserved in a 1:3 part mixture of glacial acetic acid and methanol were placed in a petri dish with glycerin and acetic acid (1:4). Petri dishes with larvae were put into larger covered petri dishes to prevent excessive evaporation, then placed in an oven at 60°C for two hours. Petri dishes were removed from the oven and left at room temperature for 22 hours. The front half of each larva was removed with forceps. The remaining rear half was placed in Hoyer's medium, prepared using the protocol in Ashburner (1989), and then incubated in an oven at 37°C for 18 hours. Slides were prepared with Hoyer's as the mounting medium with larvae oriented ventral surface up. I viewed slides under epi-illumination using a plan 16 objective, 50W mercury bulb, cross polars, a heat filter, and a green interference filter. Slides prepared in this manner should be useful for several weeks, but eventually will degrade.

## RESULTS

Sex determined by external morphology was confirmed by dissection or sexing adults in all but one larva. The only erroneously sexed larva, a *P. zelicaon*, belonged to the species most difficult to sex because larvae were light in color and the pits lacked contrasting coloration.

Sex-specific pits were clearly visible on the ventral surface of larvae (Figs. 1 and 2). Males were characterized by having one central pit on the 9th abdominal segment. Females had four pits, two located on each of the 8th and 9th abdominal segments.

For larvae requiring clearing and staining, the time necessary for the initial treatment of glycerin and acetic acid and for the Hoyer's treatment probably will depend upon the particular characteristics of the larvae to be examined. Some species may require more complete clearing and longer incubation times at perhaps greater temperatures, while others may not require clearing at all. The best preparations of

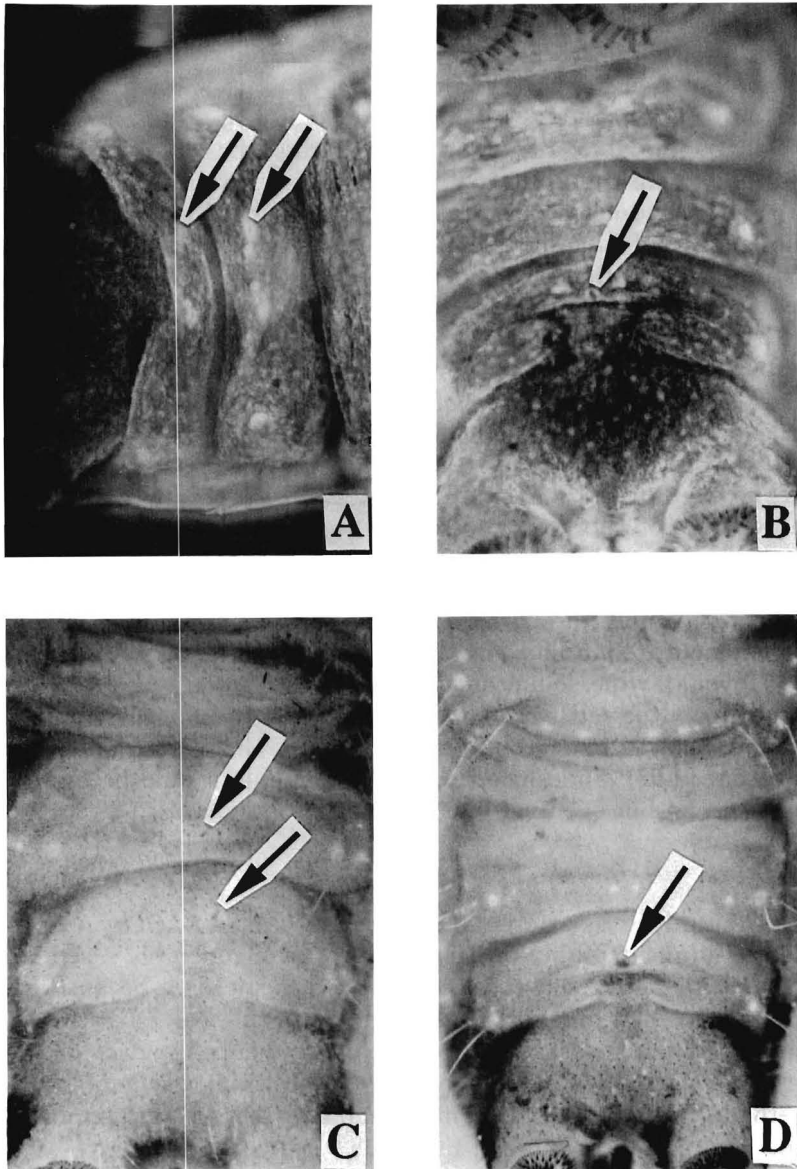


FIG. 1. First instar *Eucheira socialis* (Pieridae). (A) female (160 $\times$ ) with four pits, and (B) male (160 $\times$ ) with one pit; pits indicated by arrows. See text for details on microscope settings. Fifth instar *E. socialis*. (C) female (16 $\times$ ) with four pits, and (D) male (16 $\times$ ) with one pit. All animals in the photos are preserved and, except (A), are oriented with the posterior end downward.

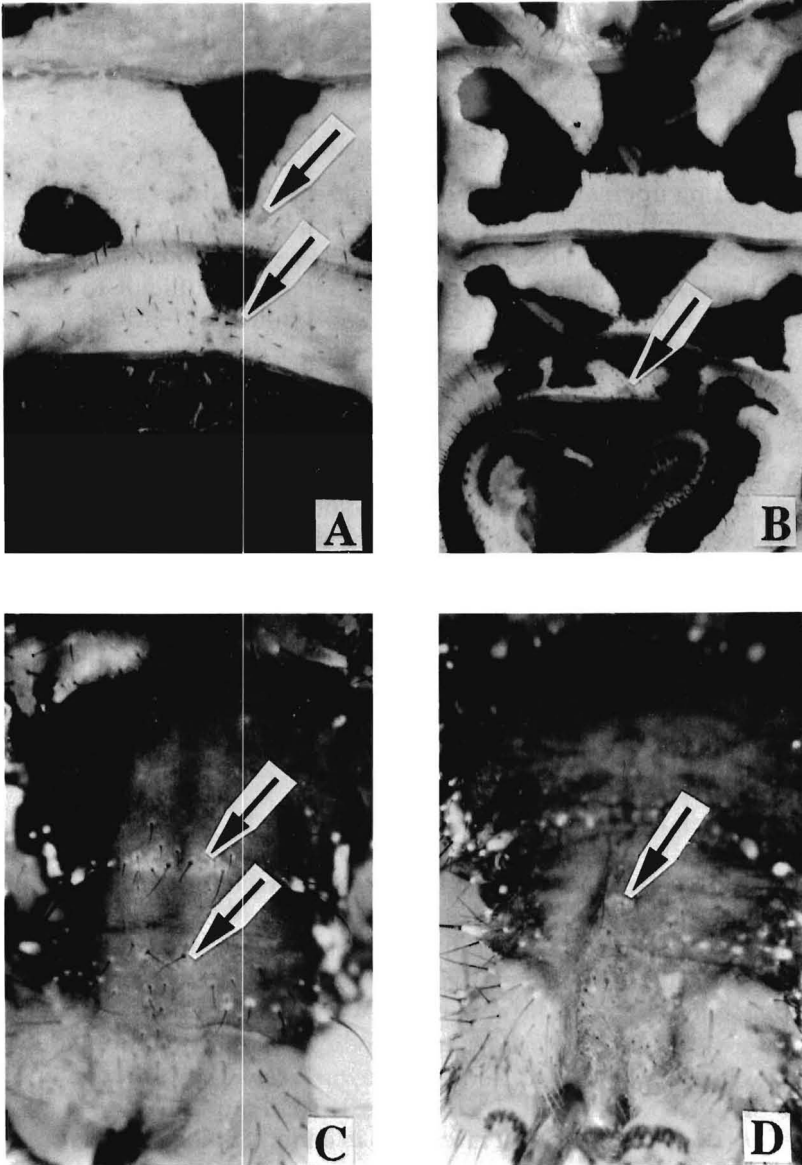


FIG. 2. *Papilio zelicaon* (Papilionidae) (A) female (20 $\times$ ), (B) male (16 $\times$ ); *Junonia coenia* (Nymphalidae) (C) female (18 $\times$ ), (D) male (20 $\times$ ). All animals in the photos are preserved and oriented with the posterior end downward.

first instar *E. socialis* were those with partial clearing; second instars could be sexed readily without clearing but needed the additional magnification of the compound microscope and epi-illumination.

#### DISCUSSION

Depending upon age and/or species, it was sometimes easier to identify one sex or the other. In *E. socialis*, female second to fourth instars were readily identified because the four pits were surrounded by lighter colored halos, while the male pits were relatively difficult to discern. Fifth and sixth instar male *E. socialis* were quickly identified because the pit typically was darkened while the female pits were less distinctive. In contrast, female *J. coenia* were much more distinctive than the males even though only one pair of pits in the female was predictably discernable. Female *P. zelicaon* also were easier to sex than males, but both were sometimes difficult because these larvae are very light in color, and the pits lacked contrasting coloration.

Lavenseau (1982) sexed first instars by searching for abdominal pits on larvae using scanning electron microscopy. This technique requires relatively elaborate protocol for preparing larvae. The technique for clearing and staining first instar larvae described here requires only a microscope capable of epi-illumination. The procedure used here for clearing larvae using Hoyer's medium is used in our introductory embryology course at the University of California at Davis to study cuticular mutations in *Drosophila* embryos; hence, it is simple enough to be done successfully by the inexperienced.

Two other techniques have been published to sex larvae with undifferentiated gonads which do not use sex-specific abdominal pits. The nuclei of many species of animals are known to contain darkly staining bodies which correlate with the presence of the Y chromosome (Smith 1945a, 1945b, Ennis 1976, Traut & Scholz 1978, Clarke 1984). Clarke (1984) identified the sex of living larvae by removing a proleg and scraping enough tissue for preparations which stain heteropycnotic bodies. However, these heteropycnotic bodies are not always evident in females in some species of Lepidoptera, or rarely, they are seen in both sexes (Traut & Mosbacher 1968, Ennis 1976, Traut 1976).

Seiler (1964), in a study on intersexes in a moth, *Solenobia triquetrella* F. R. (Psychidae), sexed larvae with undifferentiated gonads by following the gonoducts. In females, the gonoducts terminate at the 7th abdominal segment, and in males, they terminate at the 9th abdominal segment where they connect to the ectodermal anlage of the male copulation organ (the Organ of Herold). Newly hatched larvae were sexed by the presence or absence of the Organ of Herold. However,

this method cannot be used on living larvae no matter how mature, and I found the dissections tedious and difficult to perform.

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