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ORIGINAL ARTICLE

Diethe Ortius-Lechner · Markus Rüger · Tina Wanke

Sexing at the larval stage in two ant species of the Formicoxenini (Hymenoptera)

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Abstract In contrast to the many studies of external morphology of hymenopteran larvae little is known on internal morphology, and especially on the structure of the reproductive organs. This study describes a simple histological method which allows a very accurate (100%) determination of male and female individuals from the very first larval instar to the fourth instar in two formicoxenine ant species. Both female and male gonads are located between the fifth and sixth abdominal segments dorsodistal from the midgut. The male gonads are larger than the female ones throughout all larval instars, and have a heart-like shape formed by a small nucleus cell type (spermatogonia) organised into four or five lobes. In contrast, female gonads are round in cross-section and are formed by two types of cells, large nucleus cells (oogonia) and small nucleus cells (prospective follicle cells). Larval sexing is equally successful as in whole larvae when only the posterior part of the larvae is used, so that the anterior part is available for further studies on ploidy, genetics or others. Due to the accuracy and effectiveness of this method already in the first instar larvae, it is a true alternative to the methods used so far for primary sex ratio determination. All of them have higher error rates in determining sex ratio.

Keywords *Epimyrma* · *Leptothorax* · Formicidae · Larval gonad tissue · Sex determination · Histology

Introduction

The development of most social Hymenoptera is unique in that the larvae are totally dependent on the adults for feeding and general care. Consequently the external morphology of the larvae is simplified, and many basic insect structures are vestigial, i.e. legs, eyes and antennae, as is true for internal structures (Petralia and Vinson 1980). External morphology has been extensively studied on larvae of the world's ants (Wheeler and Wheeler 1976, 1986) to reveal a great diversity of forms and adornments, many of them taxonomically characteristic.

In contrast to this huge amount of knowledge accumulated on the external morphology of ant larvae, studies on the internal anatomy are still very scarce. General accounts of the anatomy of ant larvae include those of Pérez (1902), Wheeler (1910) and Brian (1977). Internal anatomy studies of larvae are known for the ants *Tapinoma erraticum* Latreille, 1798 (see Berlese 1901), *Formica rufa* Linnaeus, 1761 (see Pérez 1902), *Eciton burchelli* Westwood, 1842 (see Lappano 1958), *Neivamyrmex nigrescens* Cresson, 1872 (see Wang and Happ 1974) and *Solenopsis invicta* Buren, 1972 (see Petralia and Vinson 1980). In addition to these general accounts of internal larval morphology, other studies have focused on single organ systems, such as the alimentary canal (Nitschmann 1959), midgut (Weir 1957), hindgut (Bonavita-Cougourdan and Poveda 1972), labial glands (Emmert 1968, 1969), endocrine system (Weir 1959) and fat body (Jeantet 1969).

The aim of this study was to establish a method that allows a reliable separation of males and females throughout all larval stages in ants. Determination of sex ratios is essential in understanding how intracolonial conflicts between the reproductive individual (queen) and the workers are solved. These conflicts over sex allocation derive from the haplodiploid sex determination system in Hymenoptera, which are expected to lead to a more female biased sex ratio if the workers are in control and to an equal investment in both sexes if the queen is in control (Trivers and Hare 1976). Other methods so far used for primary sex ratio determination have relatively high errors of determination (20–40%) and have only been used either in eggs or in adults (Sundström et al. 1996).

Some authors have described larval hairs as secondary sexual characters that allow determination of the two sexes in some species (Trabert 1957) but not in others. However, to our knowledge, only two studies (Maidhof,

D. Ortius-Lechner (✉) · M. Rüger · T. Wanke
Universität Regensburg, Lehrstuhl für Biologie I,
93040 Regensburg, Germany
e-mail: diethilde.ortius-lechner@biologie.uni-regensburg.de

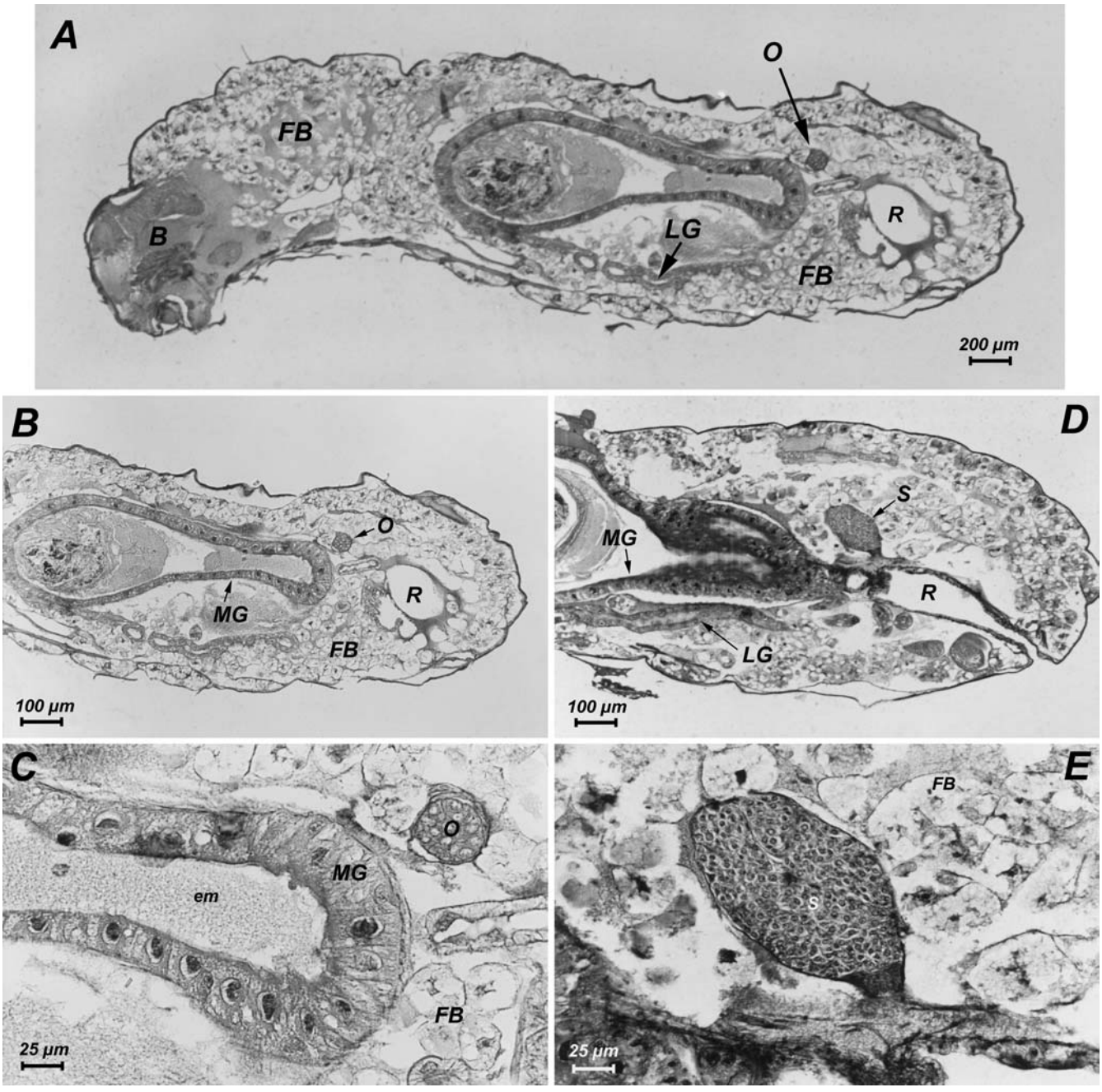

Fig. 1 **A** Longitudinal section of a fourth instar female larva of *Leptothorax acervorum*. The ovary (*O*, oogonia) is located between the fifth and sixth abdominal segments, dorsal to the midgut (*MG*). **B**, **C** Details of the area with the ovary from **A**. **D** Longitudinal section of a male fourth instar larva of *L. acervorum*. The testes (*S*, spermatogonia) are located between the fifth and sixth abdominal segments distal of the midgut. **E** Details of the testes, with their characteristic small nuclei spermatogonial cells. *B* Brain, *FB* fat body, *LG* labial gland, *R* rectum, *em* excretable mass

unpublished thesis; Petralia and Vinson 1980) have so far covered the internal anatomy of the reproductive organs of larvae in more detail. While Maidhof mentions that sex-specific gonadal tissue can already be separated from the first larval instar, gonadal tissue specificity was only described for fourth instar larvae in *S. invicta* (Petralia and Vinson 1980). Therefore, we set out to establish an easy histological characterisation of sex-specific larval gonad tissue on whole larvae of different developmental stages. In addition, the technique was improved, so that gonad tissue characterisation was also possible with halved larvae, to allow studies where the other half of the individual can be further used for complementary analysis (for example, genetic).

Materials and methods

Complete colonies of the socially parasitic ant *Epimyrma ravouxi* André, 1896 were collected together with workers of their host

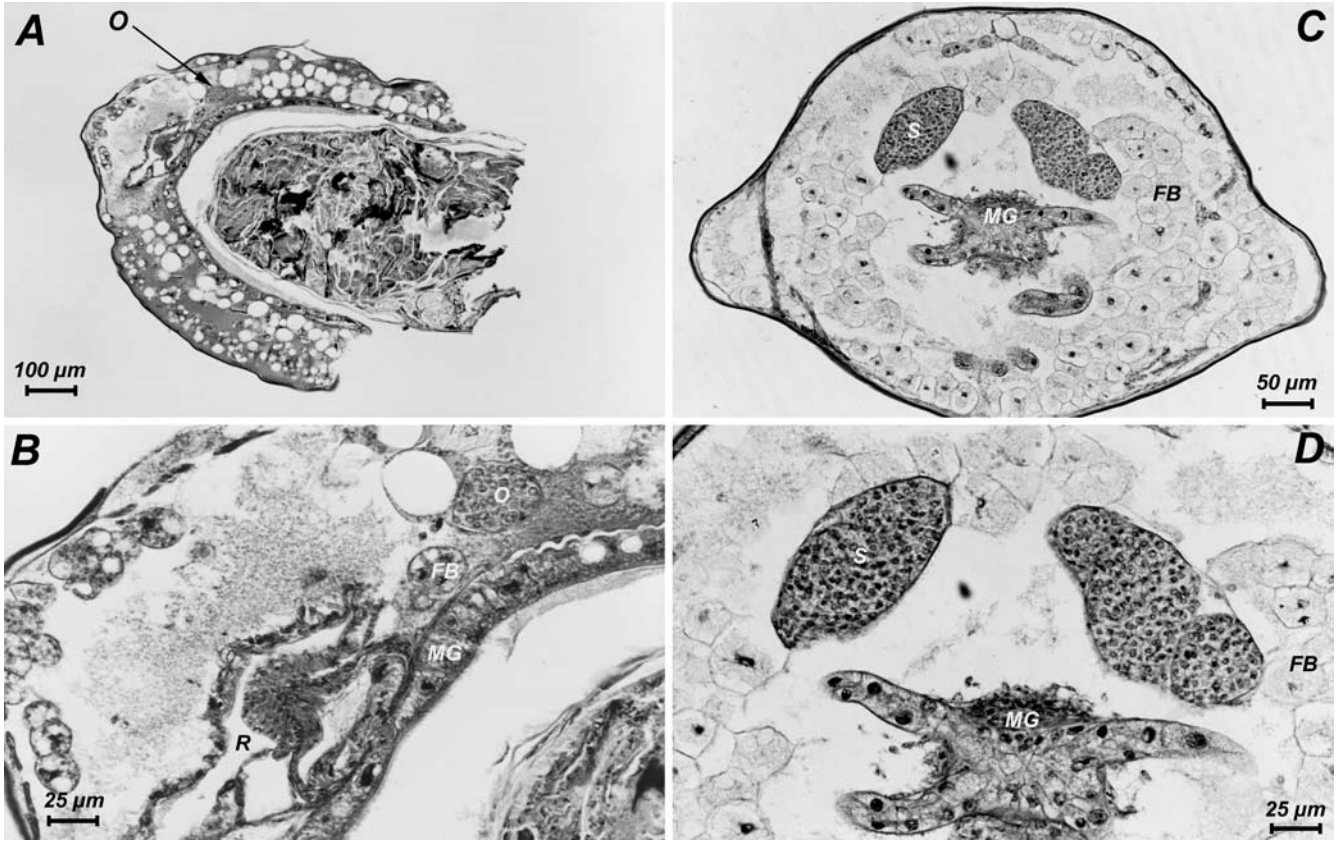

Fig. 2 **A** Longitudinal section of the posterior half of a third instar female larva of *Epimyrma ravouxi*. **B** Details of the ovarial tissue with location and characteristics equal to *L. acervorum*. **C**, **D** Cross-section of a halved second instar male larva of *E. ravouxi* with details of the lobal organisation of spermatogonial cells. Abbreviations are as in Fig. 1

species *Leptothorax unifasciatus* Latreille, 1798 and/or *Leptothorax nigriceps* Mayr, 1855 in Waldenhausen, Germany in September 2001. In addition to the *E. ravouxi* larvae, larvae of the larger, free-living *Leptothorax acervorum* Fabricius, 1793 from a population near Regensburg, Germany were used for sexing.

To ensure that the sex-specific gonads could be differentiated, first tests of the histological method described here were performed on entire larvae of *L. acervorum*. Initially, all larvae used were classified into one of the larval stages following measurement of their head capsule and were then frozen separately in an Eppendorf tube. Whole larvae were immediately fixed in Carnoy's mixture (Romeis 1989), whereas in those where only the posterior part was used for histology, the larva had to be split prior to fixation. To do this without loss of haemolymph, the larvae were positioned on an aluminium block cooled with liquid nitrogen, and were then cut in the transversal plane with a razor blade. The anterior section was refrozen at –75°C for later use, whereas the posterior section was immediately covered with fixative.

After fixation for 3 h in a likewise marked tube, both whole and half larvae were washed in 99% EtOH twice for 30 min and once for 1 h, while exchanging the ethanol. They were then soaked in methyl benzoate overnight and heated to 60°C the next day. During this process, methyl benzoate was replaced by a mixture of methyl benzoate and paraffin (1:1). After another hour, the mixture was replaced by pure paraffin (60°C) which had to be changed seven times, every half hour. Both complete larvae and the posterior parts were embedded in paraffin and sectioned with a Reichert-Jung 2040 microtome. The 7-µm sections were stained with "Delafields Hämalaun" (Romeis 1989) and viewed on a Zeiss Axiophot M45 where the pictures were also taken.

Results

This simple histological method allowed an unambiguous recognition of males and females in all larval stages of *E. ravouxi* and *L. acervorum*. Male gonads are always larger than those of females, increasing from four times larger in the first instar larvae to about eightfold in the fourth instar larvae. Histological structure of the gonads of both sexes did not differ between the two species, *L. acervorum* (Fig. 1A–E) and *E. ravouxi* (Fig. 2A–D), except that larvae of the latter species are generally smaller (and thus so are their gonads).

Female gonads

Female gonads are located between the fifth and sixth abdominal segments, dorsal from the midgut (see Fig. 1A–C for *L. acervorum* and Fig. 2A, B for *E. ravouxi*). They are characterised by two different types of cells. The majority are the oogonia with large nuclei, and there is an additional cell type with small nuclei (Figs. 1C, 2B, 3). These small nuclei cells are hypothesised to be prospective follicle cells.

First indications of a division into ovarioles become obvious in the fourth larval instar, in which the deltoid

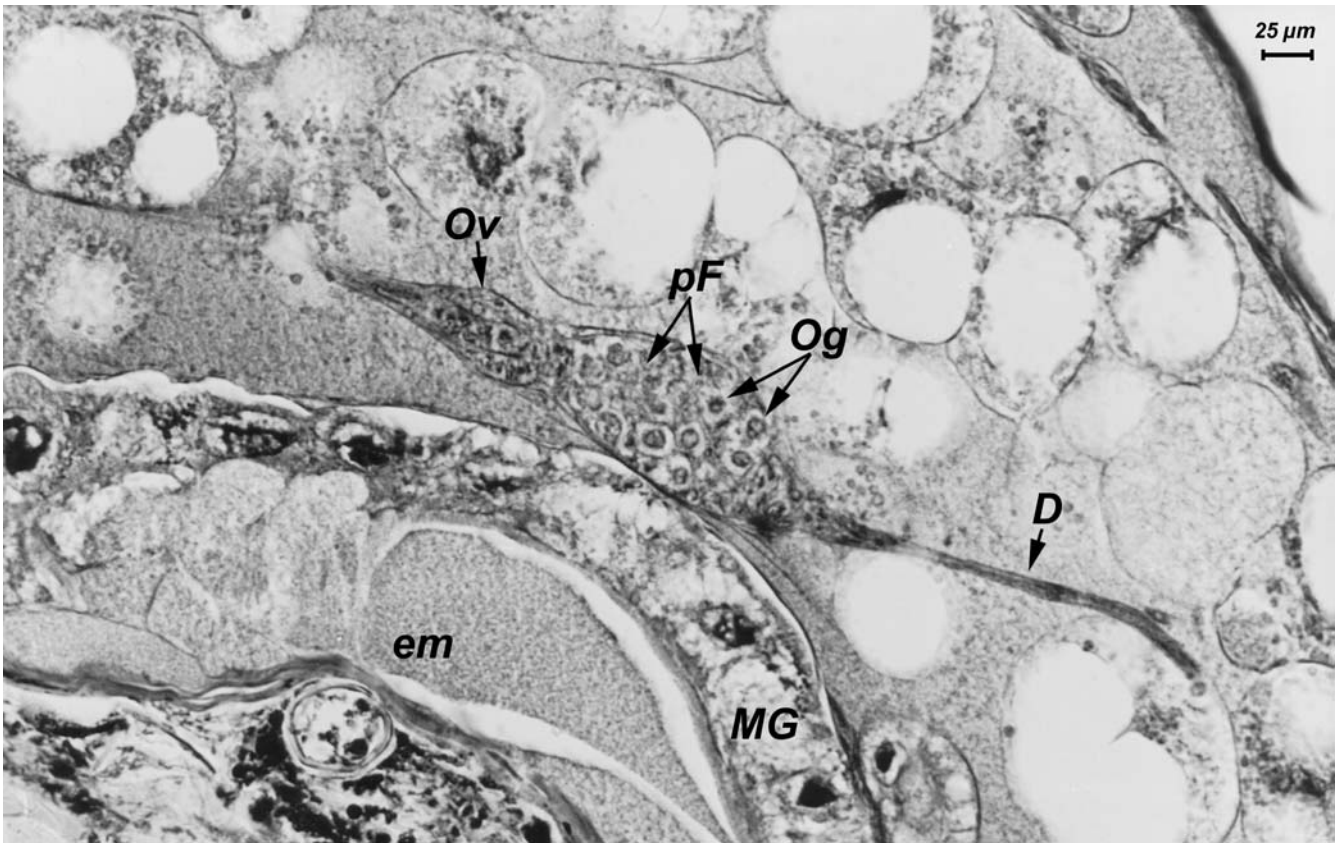

Fig. 3 Detail of a longitudinal section of a fourth instar female larva of *E. ravouxi*. The two cell types characteristic of female larval ovarian tissue (*pF* prospective follicle cells, *Og* oogonia) are highlighted in the base of the ovary which extends distally into the duct (*D*). The differentiation of ovarioles (*Ov*) is already visible

base of the ovaries continues ventrally as a thin oviduct, travelling posteriorly into the seventh abdominal segment (Fig. 3). Although the oviduct could not be traced completely, Brian (1977) states that the oviduct in ant larvae connects to the gonopodal discs.

Male gonads

The immature testes are located posterolaterally between the fifth and sixth abdominal segments. They are formed by only one cell type with small nuclei, the spermatogonia (Fig. 1D, E for *L. acervorum* and Fig. 2C, D for *E. ravouxi*), which greatly outnumber the oogonia in the female reproductive organ. The spermatogonia form four subrectangular lobes with a somewhat flat, heart-like shape. A short duct connects each lobe to a common duct which continues ventrally, ending at the connection between small intestine and rectum (Fig. 1E). Over the four larval stages the testes do not change much in form and structure but they do increase in size considerably.

Discussion

The significantly different form, size and histological organisation of male and female reproductive organs in these two species allows an easy and reliable sex determination already in the first and all the following larval stages. Males are easily recognised by heart-shaped gonads with very large tissue volume and its uniform appearance due to only one small nucleus cell type (spermatogonia). In contrast, female ovarian tissue appears small. Its form is round in cross-section and heterogeneous due to the two different cell types (large nuclei cells, oogonia, and small nuclei cells, prospective follicle cells). These findings confirm findings on the internal anatomy of reproductive organs in other species of ants (*S. invicta*: see Petralia and Vinson 1980) and seem very similar also to that described for honey bee larvae (*Apis mellifera* Linné, 1758: see Nelson 1924).

Why use this method for sexing of social insects when there are other, similarly effective methods already used? One of them, already used to determine primary sex ratios in eggs, is to squash chromosomes (Imai 1966; Sundström et al. 1996). This method is laborious and determination success is reduced to about 60%. To use genetic markers (microsatellites) would be more accurate, but with the drawback that a certain allele constellation would be necessary in the queen and her mate, so that all the diploid eggs would appear heterozygous. In addition, both of these methods will miss the occasional diploid male, which can occur in Hymenoptera, as all diploids

would be considered female due to the lack of sexing the eggs before determining ploidy. A third method has been introduced recently for sexing of Hymenopteran eggs (FISH; DeMenten, personal communication) with only 20% error but with the same drawbacks mentioned for the other two methods.

None of these methods has ever been used in the larval stages where sex is equally concealed as in eggs. The histological method introduced here not only allows sexing of larval individuals with 100% accuracy in whole larvae, but also in half of the larval tissue so that the other half (anterior part) can be used for one of the three methods mentioned above, to study ploidy, population genetics or chemistry. The histological appearance of the larval reproductive organs in both sexes does not seem species specific, so that it can be used broadly in Hymenoptera for sexing in already very early developmental stages.

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