

Research article

Larval sex identification in the paper wasp *Polistes dominulus* (Vespidae, Hymenoptera)

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Abstract. Identifying the sex of larvae is important in social Hymenoptera. Until now for *Polistes* wasps it has been necessary to genotype larvae at microsatellite loci, and assign their sex based on homozygosity at these loci. In our study on the paper wasp *Polistes dominulus* we have found morphological differences between larval sexes that can be used for larvae from the 3rd instar on to easily and cheaply identify larval sex: the external gonopore and the shape and size of larval gonads. The robustness of these indicators was supported by genotype data at four microsatellite loci. Using gonopore and gonad features for sex assignment will assign diploid males as males, unlike techniques based on genetic loci or chromosomes.

Keywords: *Polistes dominulus*, sex identification, wasp larvae, larval gonads, larval sex.

Introduction

Morphological sex identification at the larval stage would be very useful in *Polistes* wasps because they are used for work on issues like queen-worker conflict over sex ratio in Hymenoptera where sex identification is important. Such methods could then replace the more laborious and expensive method of using microsatellites, which assign sex based on homozygosity at multiple loci. In Hymenoptera females are diploid and therefore often heterozygous at highly polymorphic microsatellite loci and males are haploid and therefore hemizygous.

To date studies of larval morphology in Hymenoptera have been few. Some morphological studies have served for systematic analyses (for ants, Wheeler and Wheeler, 1976; for wasps, Yamane, 1976; Kojima, 1998; for bees,

Chauvin, 1968). Others focused on the internal anatomy of larvae (Wheeler and Wheeler, 1979, Yamane, 1976; Kojima, 1998; Dade, 1977). A recent paper (Ortius-Lechner et al., 2003) described differences in male and female reproductive organs in larvae of the ant *Leptothorax acervorum* and its social parasite *Epimyrma ravouxi*. There are a few studies which describe larval sex determination on the basis of cuticular structures in other Hymenoptera species (in *Bombus terrestris*, Duchateau and Van Leeuwen, 1990, in *Vespa analis insularis*, Yamane, 1976; in *Apis mellifera*, Santomauro and Engels, 2002). Although Lester and Selander (1981) found male and female larvae of *Polistes* to be morphologically indistinguishable, we found anatomical structures that make reliable and easy discrimination of male and female larvae possible, as is described in this paper.

Materials and methods

Near Florence, Italy, colonies of *Polistes dominulus* are begun in March by 1–7 mated females. They lay eggs that develop into larvae, then pupae, then emerge as female workers beginning in late May or early June (Pardi, 1942).

Larvae collection

a) Larvae of known sex:

In May 2004 we collected 8 pre-emergence colonies of *Polistes dominulus* near Florence, Italy (Table 1). From five of these nests we immediately collected 15 last instar larvae (5th instar) that are highly likely to be all females at this time (Pardi, 1942). After the first workers emerged, we removed the queens from the other three nests housed in laboratory. A month later, we collected 20 male larvae produced by unmated workers from these orphaned nests (Strassmann et al., 2004).

b) Larvae of unknown sex:

In July 2004, we collected 9 *P. dominulus* colonies likely to contain both male and female larvae. We analyzed 70 last instar larvae from these

Table 1. Summary table of larval collections and correspondence of sex assignment between the three methods.

Collection Date	# colonies	Larval condition	Instar	Sex	# by gonads	# by pore morphology	# by micro-satellites	# by season or orphan state
May 2004	5	Fresh	Last	Female	15	–	–	15
May 2004	3	Fresh	Last	Male	20	–	–	20
July 2004	9	Fresh	Last (tot 70 larvae)	Male	51	–	49	–
July 2004	9	Fresh	Last (tot 70 larvae)	Female	19	–	16	–
July 2005	15	Fresh	Last (tot 48 larvae)	Male	27	27	–	–
July 2005	15	Fresh	Last (tot 48 larvae)	Female	21	21	–	–
July 2005	5	Frozen	2 nd (tot 24 larvae)	?	–	Not evident	–	–
July 2005	5	Frozen	3 rd (tot 23 larvae)	Male	–	5	–	–
July 2005	5	Frozen	3 rd (tot 23 larvae)	Female	–	8	–	–
July 2005	5	Frozen	4 th (tot 15 larvae)	Male	–	9	–	–
July 2005	5	Frozen	4 th (tot 15 larvae)	Female	–	6	–	–
July 2005	5	Frozen	Last (tot 30 larvae)	Male	–	18	–	–
July 2005	5	Frozen	Last (tot 30 larvae)	Female	–	12	–	–

colonies (Table 1). In July 2005 we collected 15 *P. dominulus* colonies and we extracted from them 48 last instar larvae. Another 5 colonies collected in the same period were frozen immediately. We removed 92 larvae of the 2nd, 3rd, 4th and last instars from the these frozen nests, sorting them according to Kojima (1998) to see how early we could use gonadal pores to assign sex.

We extracted all the larvae from their cells using soft tweezers and anesthetized living larvae for dissection by putting them in the freezer for few minutes before dissection.

Dissection and preparation of the specimens

We dissected larvae under a stereo microscope beginning with a short vertical cut in the dorsal cuticle at the 8th abdominal segment. We put one or two drops of Toluidine Blue (1%) into the cut and waited a few seconds. Toluidine Blue is a not selective stain used to detect whole organs in their context: the gonads absorb much less colour than the surrounding tissues. Previous work indicated that this technique is only useful on fresh, not frozen or otherwise preserved specimens. We first dissected the larvae of known sex (females from the pre-emergence nests and males from the orphaned nests), and took pictures of all larval gonads under a microscope at 25 magnification. Then we dissected 70 last instar larvae of unknown sex from 9 post-emergence colonies. We measured the width and the length of a single gonadal packet for 26 samples. We statistically compared gonad size of males and females using the Mann-Whitney *U*-test. After dissection the 70 larvae were preserved in 96% ethanol and genotyped.

We also took several measurements of the head (head capsule area, length, width, distance between the antennae, mandible length) but since we did not find any of these characters useful in distinguishing males from females, this work is not reported further.

External morphology

We separated the 48 larvae from the 15 colonies of July 2005 by sex on the basis of their stained gonads and put them in 96% alcohol. Immediately and after one week we looked at the cuticular genital appendages, following reports for other species of social Hymenoptera (Duchateau and Van Leeuwen, 1990; Yamane, 1976).

We placed the larvae of the other 5 nests of 2005 in 96% alcohol without staining the gonads first. We checked daily to see how long it would take for the gonadal pores to be sufficiently fixed to be identifiable.

Genetical analysis

As a check on the sex of 70 dissected larvae we conducted genetical analysis using four highly polymorphic loci (Pdom 7, 140, 122, 117) previously reported for a different *P. dominulus* population located near Florence (Henshaw, 2000). We evaluated the degree of heterozygosity at these four loci since males are haploid and so should never have more than a single allele at any locus. The four loci we used, have expected heterozygosities of 0.73, 0.85, 0.85, and 0.83 respectively (Henshaw, 2000). We estimate the probability of a female being homozygous at all four loci as the product of 1 minus the heterozygosity level of the four loci. This is: $(1-0.73)(1-0.85)(1-0.85)(1-0.83)$ or 0.001033. Thus there is about a one in a thousand chance that an individual showing a single allele at all four loci is a homozygous female and not a haploid male.

In order to extract the DNA a piece of tissue from each larva was cut and placed in an empty vial for 24 hours, to let the ethanol evaporate. The tissue was added to 150 μ l of 5% Bio-Rad Chelex-100 and 10 μ l proteinase K (20 mg/ml), in a 1.5 ml Eppendorf tube. The sample was incubated at 56°C for 2 hours. The tube was then vortexed for 10 seconds, boiled for 8 minutes, then vortexed and centrifuged for 3 min at 12000 rpm. The four microsatellites were amplified through PCR in each DNA sample with fluorescent primers. The PCR product was analyzed using an ABI 3100 Genetic Analyzer, which detected the length of each allele.

Results

Gonadal morphology and size

The larvae from orphaned colonies matched the hemizygous larvae and can be considered to be males. Male gonads become immediately visible after staining with Toluidine. Male gonads have two symmetric rectangular packets containing three cylindrical glands each (Fig. 1a, below). These three glands are surrounded by a tightly attached tunic. The gonads sometimes appear to be slightly bent, following the contours of the intestine.

The larvae from the pre-emergence colonies matched the heterozygous larvae and can be considered to be females. Female gonads appear slightly later against the Toluidine blue background. They are smaller and closer to the surface of the intestine than are male gonads. They

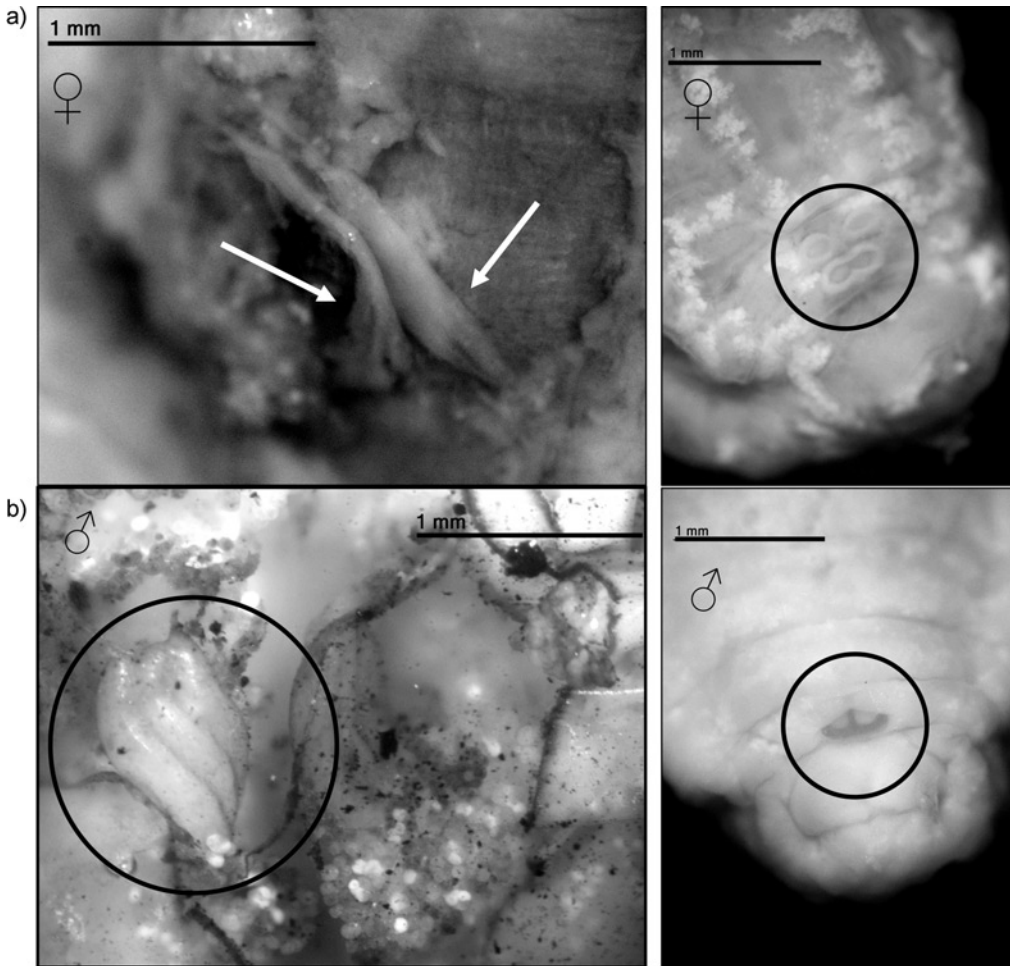


Fig. 1 a, gonads: above, female; below, male. b, gonopores: above, female; below, male.

Table 2. Average size (\pm SD) of the tunic and the gonad of male and female larvae

	Female gonads (N) mean \pm SD (mm)	Male gonads (N) mean \pm SD (mm)	Mann-Whitney test	P exact
Length of tunic	(9) 1.46 \pm 0.40	(13) 1.36 \pm 0.40	U=45.00	n.s.
Width of tunic	(10) 0.21 \pm 0.05	(14) 0.46 \pm 0.11	U=1.00	<0.001
Length of gonad	(12) 0.65 \pm 0.25	(10) 1.01 \pm 0.29	U=19.50	0.006
Width of gonad	(12) 0.04 \pm 0.01	(11) 0.17 \pm 0.04	U=0.00	<0.001

are found in the adipose cell layer and differ from the male gonads in size and shape (Fig. 1a, above). The envelope containing the female gonads is usually narrower at the anterior end, presenting a droplet shape. The packets are connected by a ligament that elongates in the anterior direction. Once the tunic that surrounds the gonads is removed, these are visible as two groups of glands, each constituted by three thin cylinders attached to the tunic. Male gonads are significantly larger than female, allowing a rapid distinction between the two sexes (Table 2).

External morphology

On the ventral abdominal surface there is a transparent circular area containing the genital appendages. In female larvae there is a pair of small circles on the 8th segment, and an unpaired disc with 2 circles inside on the 9th segment (Fig. 1b, above), whereas in male larvae only the unpaired disc on the 9th segment is present (Fig. 1b, below). In 2004 we examined the gonopores in 48 larvae (27 male and 21 female larvae) after one week in alcohol preservation. In all cases, gonopores matched with gonads respect to the predicted sex (Table 1).

In 2005 we further investigated larval gonopores using the sample of 92 frozen larvae from 5 nests (30 of the 5th instar, 15 of the 4th instar, 23 of the 3rd instar and 24 of the 2nd instar). The gonopores become visible from the 4th day after alcohol fixation in the 5th, 4th and 3rd instars, while in younger larvae they were not easily visible (Table 1).

Genetical analysis

Fifty-one of the larvae had a single allele at all four loci indicating they were male hemizygotes (Table 1). The remaining 19 larvae were heterozygotes, indicating they were female. The genetical analyses matched the sex determined by gonadal staining for 65 of the 70 larvae (92.9%). The anatomical sex determination of five larvae did not fit results of genetic analyses: 3 larvae sexed as females resulted to be males and 2 larvae sexed as males resulted to be females for genetic analysis.

Discussion

Here we present a method for distinguishing male and female larvae in the paper wasp *Polistes dominulus* based on larval gonadal morphology and gonopore structure. Genital staining has the advantage of rapidity: the stain works immediately on fresh specimens. Examination of the gonopore requires a four day wait for fixing the tissues in alcohol, but this technique is better for specimens that have been frozen.

Even if we explore larval sex identification using gonadal morphology and gonopore structure only in *Polistes dominulus*, it is possible that the same methods can be used for other species of *Polistes* wasps. In fact, gonopores have also proven to be useful character for sex differentiation in other social Hymenoptera: *Bombus terrestris* (Duchateau and Van Leeuwen, 1990) and *Vespa* spp. (Yamane, 1976). However, it was not easy to see these cuticular structures in *P. dominulus* larvae before the third instar. This is different from *Bombus terrestris* in which males and females can be distinguished even as first instar larvae (Duchateau and Van Leeuwen, 1990). In 3rd instar larvae the gonopores are not always visible because the previous ecdysis sometimes covers the gonopore zone though this tegument can sometimes be removed. Our methods do not work to sex the larvae before the third instar, however, they can be used in studies where late brood sex determination is required.

Other methods of assessing sex exist, but are not as quick or cheap. They include observing chromosome number (Ueno and Tanaka, 1997), fluorescence *in situ* hybridization (FISH) (de Menten et al., 2003), flow

cytometry analysis (Aron et al., 2003), and microsatellite genetic analysis (Arévalo et al., 1998). These methods all depend on males being haploid, as all normal males are. Our methods would assign diploid males to be male, unlike the other ones.

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