Insect. Soc. (2014) 61:51–55
DOI 10.1007/s00040-013-0323-5

Insectes Sociaux

RESEARCH ARTICLE

A non-destructive method for identifying the sex of ant larvae

C. A. Penick · J. Ebie · D. Moore

Received: 1 August 2013 / Revised: 26 September 2013 / Accepted: 10 October 2013 / Published online: 25 October 2013

Abstract The differences between adult male and female ants are often striking and obvious, yet both sexes appear virtually identical at the larval stage. Current methods for determining larval sex rely on genetic analyses or histology, both of which require killing all larvae examined. Here, we describe a method for identifying larval sex in vivo based on visible differences in genital imaginal discs. Using a light microscope, clear differences in genital disc morphology were observed between male and female larvae of the ponerine ant *Harpegnathos saltator*. Next, we investigated whether this technique could be broadly applied within ants and found similar differences in genital discs between male and female larvae of *Aphaenogaster cockerelli* and *Camponotus floridanus*. Taken together, our results show that genital discs can be used as a reliable indicator of larval sex in species from at least three major ant subfamilies. This technique should facilitate research into topics where information about larval sex is required.

Keywords Sex identification · Larval morphology · Genital discs · Development · Research methods

Introduction

The larvae of social insects are dependent on adult care throughout their development and have reduced morphological features. This simplification of body structure has made it difficult to distinguish larval sex in research settings. In a few cases, larvae display sex-specific or caste-specific patterns in external morphology (Edwards, 1991; Solis et al., 2010; Penick et al., 2012a), but most morphological differences associated with sex are internal and related to gonadal tissue (Petralia and Vinson, 1980). As a result, methods for determining larval sex have relied on histology (Ortius-Lechner et al., 2003) or tissue fixation and injection with dyes that make internal structures visible (Duchateau and van Leeuwen, 1990; Cotoneschi et al., 2007). An alternative approach has used genetic techniques that take advantage of haplodiploid sex determination in social Hymenoptera to establish larval sex based on ploidy (Aron et al., 2003; Cotoneschi et al., 2007). While these methods have certain advantages, one major drawback is that they require killing larvae and sacrificing tissue that could be used for other analyses. This precludes studies that require live larvae of known sex, or studies where whole-body tissue analysis may be important (e.g. transcriptomics or hormone quantification).

Unlike adults, larval cuticle is generally transparent, and some internal structures are visible in live individuals. One example is the genital discs, which are imaginal cells that eventually proliferate into the adult sex organs during the pupal stage. Evidence from wasps and bees suggests that genital discs can be used to distinguish male and female larvae after larvae have been injected with dye (Duchateau and van Leeuwen, 1990; Cotoneschi et al., 2007), and we predicted that in some species these discs may be visible directly through the larval cuticle. We investigated genital discs in live larvae of three ant species (*Harpegnathos saltator*, *Aphaenogaster cockerelli*, and *Camponotus floridanus*) and describe how genital disc morphology can be used to determine larval sex. This technique provides a

C. A. Penick · J. Ebie · D. Moore
School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501, USA

C. A. Penick (✉)
Department of Biology, North Carolina State University, Raleigh, NC 27695-7617, USA
e-mail: clintpenick@gmail.com

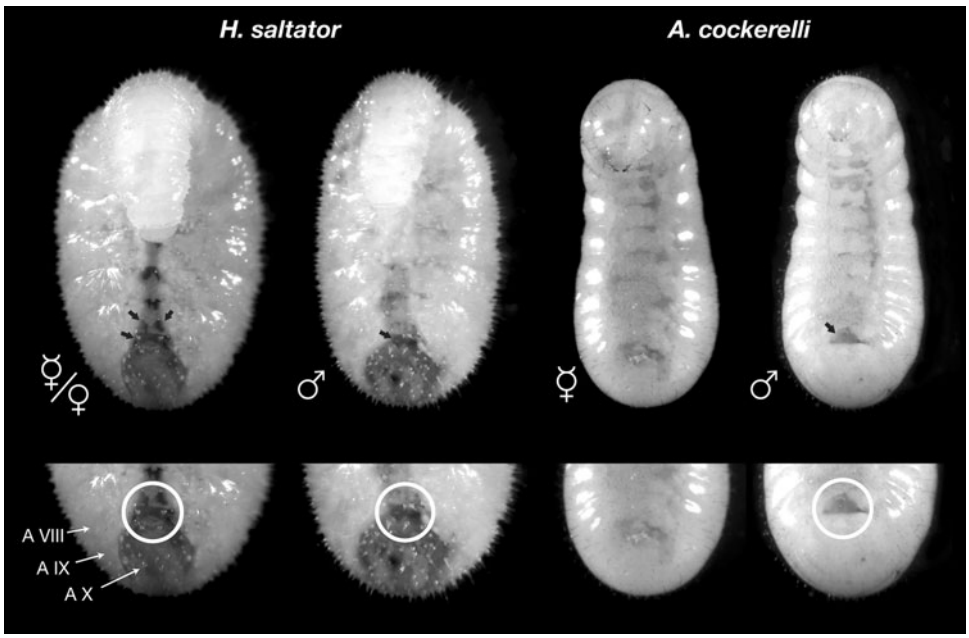

Fig. 1 Location and morphology of genital discs. Female larvae of *H. saltator* display a characteristic pattern with two rounded discs present above a larger "window" on abdominal segment *A–IX* that contains two large, adjacent discs (*arrows* indicate discs on top images). The discs are nearly transparent, so they can be identified as clear regions that are outlined by surrounding fat cells (see Fig. 2 for a schematic representation). Males of *H. saltator* and *A. cockerelli* only have the lower disc pattern, visible just above segment *A–X*, while female workers of *A. cockerelli* have no visible discs. Instead, fat cells obscure the location where the "window" is visible in males of *A. cockerelli*. The pattern in *C. floridanus* is similar to that pictured for *A. cockerelli* and is depicted in Fig. 2. Larval depictions are not to scale, but male and female larvae are similar in size within each species

reliable and rapid method to identify larval sex in ants and does not require destructive sampling.

Methods

Study species

All examinations were performed using laboratory colonies of *H. saltator*, *A. cockerelli* (previously *Novomessor cockerelli*), and *C. floridanus*. These species are representatives from three major ant subfamilies (Ponerinae, Myrmicinae, and Formicinae), and methods for identifying larval sex in these species should be broadly applicable to other ant species. Colonies of *H. saltator* were originally collected from India, while colonies of *A. cockerelli* and *C. floridanus* were collected from Arizona and Florida (USA), respectively. In the laboratory, colonies were housed in plastic nest boxes with a plaster floor and maintained at 25 °C under a 12:12 light/dark cycle (for additional information about collection and rearing conditions see Penick et al. (2012b), Smith et al. (2008a), and Moore and Liebig (2010)).

Identifying larval sex

To investigate differences associated with larval sex, larvae of known sex were examined using a light microscope (Leica MZ9.5, up to 60× magnification) with a fibre optic light source. Female worker larvae were taken from colonies exclusively rearing workers (i.e. only worker pupae were present), and male larvae came from colonies of unmated workers, which exclusively lay male-destined, haploid eggs. Because major differences in body shape or hair morphology were not apparent, we focused on differences associated with genital disc morphology [sometimes referred to as gonopodal of gonopore discs (Petralia and Vinson, 1980; Cotoneschi et al., 2007)]. Genital disc tissue was located on the ventral-posterior region of larvae (abdominal segments VIII and IX) directly below the cuticular surface. Genital discs were slightly opaque compared to surrounding hemolymph and were visible inside a "window" clear of fat cells (Fig. 1). To visualize the discs more clearly, it helped to aim the light sources towards either side of the larva rather than from directly above. In sexes where genital discs were not clearly observed, dense fat deposits generally occupied the area where discs would have been located. We only observed genital discs in late-instar larvae (e.g. fourth-instar larvae in *H. saltator*), so early instars were excluded from our analysis.

To determine whether genital disc morphology could be used as a reliable indicator of sex, groups of late-instar larvae were sorted based on their discs characteristics. In *H. saltator*, two distinct genital disc patterns were present (Figs. 1, 2). Larvae were sorted based on these differences

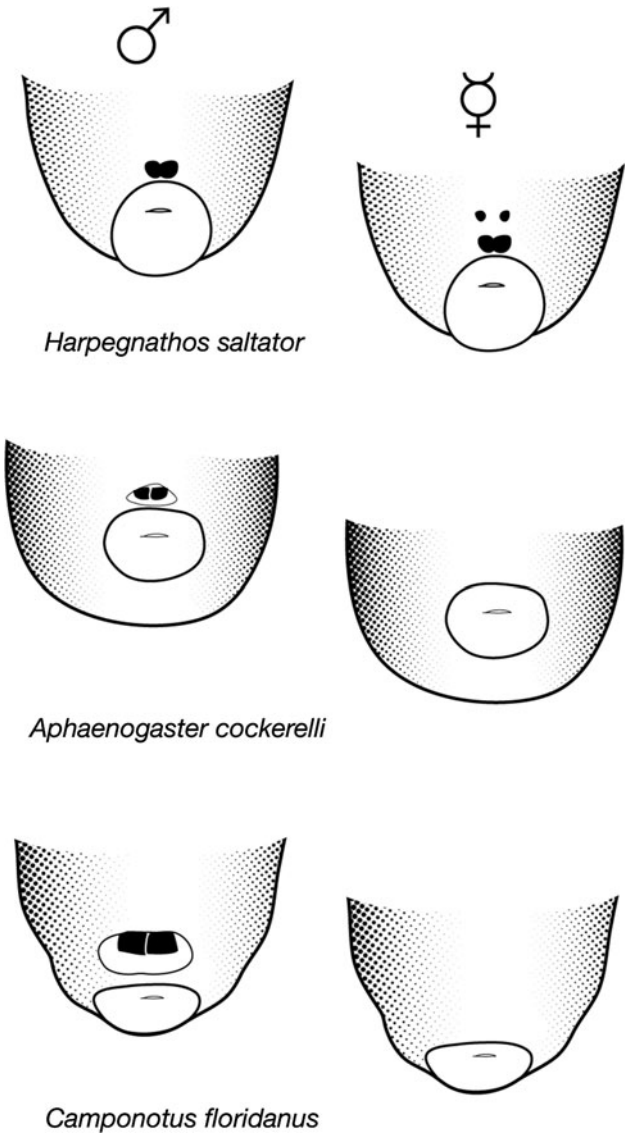

Fig. 2 Visible differences in genital disc morphology between live male and female worker larvae. Areas where discs are not present are generally filled with opaque fat cells

and divided between two separate adult worker groups. Sex was confirmed when larvae reached the pupal stage, at which point sex could be clearly assessed based on pronounced morphological differences between male and female pupae. A similar technique was used for *A. cockerelli* and *C. floridanus*; however, male larvae were taken from isolated worker groups where un-mated workers laid haploid eggs that developed exclusively into males. Female larvae were taken from queenright colonies not producing sexuals. In all groups examined, we specifically chose male and female worker larvae that overlapped in size. Larvae were mixed randomly between colonies so that sex was not known before sorting. Again, sex was determined at the pupal stage. To test whether our predictions of larval sex were significantly different from random chance, we performed a χ^2 analysis using contingency tables of our results for each species.

The larval instars of *H. saltator* have been previously described (Penick et al., 2012b), and fourth-instar larvae were used for this study. For the other two species, we measured the total range of larval lengths present in the colony (excluding female sexuals) and the length of all larvae that were sorted by sex. These measurements provided information about the proportion of larvae for which sex could be determined based on genital disc morphology. Larval length was determined as a straight line along the anteroposterior axis, and measurements were calculated from photographs using ImageJ software (version 1.44, 2010). In addition to worker larvae, female sexual larvae were observed in *H. saltator* ($N = 25$) and *C. floridanus* ($N = 3$). Larvae identified as sexuals were noticeably larger than either worker or male larvae (see Penick and Liebig, 2012). Because these larvae could be easily distinguished by size, we did not include them in our sorting experiment where differences were assessed based solely on genital discs.

Results and discussion

Genital disc morphology served as a reliable indicator of larval sex in all three ant species that were investigated (Table 1). In *H. saltator*, fourth-instar female larvae displayed a characteristic genital disc pattern with two round discs on abdominal segment VIII and two abutting discs on segment IX (Fig. 2). Female larvae of *H. saltator* remain bipotential until the final days of larval life (Penick et al., 2012b), and consistent with this pattern, female sexual larvae ($N = 25$) displayed the same genital disc morphology as workers. In contrast, males possessed genital discs on segment IX, but none were visible on segment VIII (Figs. 1, 2). Where discs were not present on male larvae, the area where discs would have been located was generally clouded by fat cells (Fig. 1). Using these characters in *H. saltator*, we could predict larval sex with 100 % accuracy as determined at the pupal stage (Table 1) and sort larvae according to sex at a rate of 10–20 larvae/min.

Male and female worker larvae of *A. cockerelli* and *C. floridanus* could also be sorted based on genital disc morphology, but the pattern differed from that found in *H. saltator*. Males possessed two genital discs located inside a "window" clear of fat cells on abdominal segment IX. In contrast, no genital discs were visible in female worker larvae. A small proportion of larvae were misidentified in *A. cockerelli* (Table 1), but our experience with *H. saltator* suggests that accuracy could be improved with increased experience identifying larval sex. Our major focus for this study was discriminating between male and female worker larvae because both types overlapped in size. However, we

Table 1 Accuracy of larval sex identification method

Species	Predicted sex	Total number enclosed		Accuracy (%)*	Larval length (range in mm)	
		Male	Female (worker)		All larvae	Larvae identified[a]
Harpegnathos saltator	Male	37	0	100	1.3–6.5	4.1–6.5
	Female	0	73	100		
Aphaenogaster cockerelli	Male	23	3	88	1.0–6.2	4.1–6.2
	Female	2	20	91		
Camponotus floridanus	Male	9	0	100	1.1–7.7	5.2–7.7
	Female	0	22	100		

* For all species, our ability to determine larval sex was significantly compared to random chance (χ^2, $p < 0.0001$)

[a] Represents the subset of larval sizes we actually identified as the subset of total larval lengths present in each species

did observe a limited number of female sexual larvae in *C. floridanus* ($N = 3$). Female sexual larvae were approximately 2–3× larger than worker or male larvae. At this stage, their hemolymph was completely clouded by fat cells, and no genital discs were visible, which was similar to worker-destined larvae. The genital disc morphology of female sexuals in *H. saltator* was also similar to workers, and this appears to be the case for *Solenopsis invicta* as well (Petralia and Vinson, 1980). Nevertheless, future applications of this technique to other species should investigate whether differences in genital disc morphology occur between female worker and female sexual larvae.

Compared to other methods of larval sex identification, the technique described here facilitates identification of live larvae. This has several advantages over methods that rely on histology or genetic sampling. First, larvae can be sorted rapidly without need for specialized equipment or supplies. Second, diploid males could likely be identified (see Cotoneschi et al., 2007) even when genetic tests may not distinguish these from females (Santomauro and Engels, 2002). Finally, because larvae remain alive, they can be used in subsequent studies where larvae of known sex are desired. This technique should also have broad application to other ant species: we found sex-specific differences in genital disc morphology from species representing three major ant subfamilies (Ponerinae, Myrmicinae, and Formicinae), and genital disc morphology also differs between male and female larvae in *S. invicta* (Petralia and Vinson, 1980), *Pheidole bicarinata* (Wheeler, 1982), as well as bumble bees and wasps (Yamane, 1976; Duchateau and van Leeuwen, 1990; Cotoneschi et al., 2007).

In bumble bees, genital discs are visible in the earliest instars (Duchateau and van Leeuwen, 1990), but we were only able to discern genital disc morphology in late-instar larvae in this study. This limits the application of our method, but the last instars generally take up the longest proportion of the total larval period (Baratte et al., 2005; Penick et al., 2012b). Early larval instars are often fleeting, lasting anywhere from one to several days (Wheeler, 1990; Penick et al., 2012b), while the bulk of larval growth occurs during the last larval stages when genital discs are visible. Regulation of caste determination may also be the most important during late larval stages, including queen determination (Brian, 1973; Penick and Liebig, 2012) as well as soldier determination (Wheeler and Nijhout, 1981; Rajakumar et al., 2012). For example, in *H. saltator* the duration of the last larval instar makes up 40 % of the total larval period, and this is the most important period for the regulation of queen determination (Penick and Liebig, 2012; Penick et al., 2012b). If necessary, it may be possible to view imaginal discs in early instars by soaking larvae in a clearing solution (e.g. Petralia and Vinson, 1980), but this would obviously kill the larvae and damage specimens for gene expression studies.

This technique should have applications for future studies that investigate larval development. This is especially relevant for studies on caste determination where male larvae should be excluded. There has been increased interest in elucidating the genetic factors underlying queen determination (Smith et al., 2008b), and these studies often require whole larvae for analyses. Additionally, juvenile hormone (JH) has been found to be an important regulator of caste determination (Wheeler, 1986), but there has yet to be a study that investigates actual JH levels or other hormones in female sexual larvae compared to workers. These types of studies would benefit from a method to reliably exclude males. While our technique allows researchers to distinguish larval sex, it is still largely unclear how ants discriminate the sex of their own brood (Nonacs and Carlin, 1990). Future studies on larval pheromones should benefit from methods that allow larval sex identification, including studies that investigate worker manipulation of sex ratios (e.g. Passera and Aron, 1996). Overall, this technique should open up opportunities for new research in areas that require information about larval sex where whole specimens are needed.

Acknowledgments We would like to thank Jürgen Liebig for laboratory space and access to colonies used in this study. Additionally, Kevin Haight provided assistance with colony maintenance and Adrian Smith originally collected colonies of *A. cockerelli*.

References

Aron S., De Menten L. and Van Bockstaele D. 2003. Brood sex ratio determination by flow cytometry in ants. *Mol. Ecol. Notes* **3**: 471-475

Baratte S., Cobb M., Deutsch J. and Peeters C. 2005. Morphological variations in the pre-imaginal development of the ponerine ant *Diacamma ceylonense*. *Acta Zool.* **86**: 25-31

Brian M.V. 1973. Caste control through worker attack in the ant *Myrmica*. *Insect. Soc.* **20**: 87-102

Cotoneschi C., Scognamiglio F., Scala C., Cervo R., Strassmann J.E. and Turillazzi S. 2007. Larval sex identification in the paper wasp *Polistes dominulus* (Vespidae, Hymenoptera). *Insect. Soc.* **54**: 132-135

Duchateau M. and van Leeuwen P. 1990. Early sex determination in larvae of *Bombus terrestris*. *Insect. Soc.* **37**: 232-235

Edwards J.P. 1991. Caste regulation in the pharaoh's ant *Monomorium pharaonis*: recognition and cannibalism of sexual brood by workers. *Physiol. Entomol.* **16**: 263-271

Moore D. and Liebig J. 2010. Mixed messages: fertility signaling interferes with nestmate recognition in the monogynous ant *Camponotus floridanus*. *Behav. Ecol. Sociobiol.* **64**: 1011-1018

Nonacs P. and Carlin N.F. 1990. When can ants discriminate the sex of brood? A new aspect of queen-worker conflict. *Proc. Natl Acad. Sci. USA* **87**: 9670-9673

Ortius-Lechner D., Rüger M. and Wanke T. 2003. Sexing at the larval stage in two ant species of the Formicoxenini (Hymenoptera). *Zoomorphology* **122**: 41-45

Passera L. and Aron S. 1996. Early sex discrimination and male brood elimination by workers of the Argentine ant. *Proc. R. Soc. London B* **263**: 1041-1046

Penick C.A. and Liebig J. 2012. Regulation of queen development through worker aggression in a predatory ant. *Behav. Ecol.* **23**: 992-998

Penick C.A., Copple R.N., Mendez R.A. and Smith A.A. 2012a. The role of anchor-tipped larval hairs in the organization of ant colonies. *PLoS One* **7**: e41595

Penick C.A., Prager S.S. and Liebig J. 2012b. Juvenile hormone induces queen development in late-stage larvae of the ant *Harpegnathos saltator*. *J. Insect Physiol.* **58**: 1643-1649

Rajakumar R., San Mauro D., Dijkstra M.B., Huang M.H., Wheeler D.E., Hiou-Tim F., Khila A., Cournoyea M. and Abouheif E. 2012. Ancestral developmental potential facilitates parallel evolution in ants. *Science* **335**: 79-82

Petralia R. and Vinson S.B. 1980. Internal anatomy of the fourth instar larva of the imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). *Int. J. Insect Morphol. & Embryol.* **9**: 89-106

Santomauro G. and Engels W. 2002. Sexing of newly hatched live larvae of the honey bee, *Apis mellifera*, allows the recognition of diploid drones. *Apidologie* **33**: 283-288

Smith A.A., Hölldobler B. and Liebig J. 2008a. Hydrocarbon signals explain the pattern of worker and egg policing in the ant *Aphaenogaster cockerelli*. *J. Chem. Ecol.* **34**: 1275-1282

Smith C.R., Toth A.L., Suarez A.V. and Robinson G.E. 2008b. Genetic and genomic analyses of the division of labour in insect societies. *Nature Rev. Gen.* **9**: 735-748

Solis D., Fox E., Rossi M. and Bueno O. 2010. Description of the immatures of *Linepithema humile* Mayr (Hymenoptera: Formicidae). *Biol. Res.* **43**: 19-30

Wheeler D.E. 1982. Soldier determination in the ant, *Pheidole bicarinata*. PhD-dissertation. Durham, NC: Duke University

Wheeler D.E. 1986. Developmental and physiological determinants of caste in social Hymenoptera: evolutionary implications. *Am. Nat.* **128**: 13-34

Wheeler D.E. 1990. The developmental basis of worker polymorphism in fire ants. *J. Insect Physiol.* **36**: 315-322

Wheeler D.E. and Nijhout H.F. 1981. Soldier determination in ants: new role for juvenile hormone. *Science* **213**: 361-363

Yamane S. 1976. Morphological and taxonomic studies on vespine larvae, with reference to the phylogeny of the subfamily Vespinae (Hymenoptera: Vespidae). *Insecta Matsumurana* **8**: 1-45