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TECHNICAL NOTE

Brood sex ratio determination by flow cytometry in ants

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Abstract

Sex ratio variations during brood development have important implications for the study of sex allocation in haplodiploid insects. So far, few studies have addressed this question because of the difficulty to determine the sex of the brood. We used flow cytometry to differentiate haploid males from diploid females in the ant *Linepithema humile*. Our data show that flow cytometry can be used successfully to distinguish between male and female brood on the basis of their DNA content, from the very first larval stage. Moreover, we show that flow cytometry allows sex brood determination in other ant species, as well as in nonsocial Hymenoptera.

Keywords: ants, flow cytometry, haplodiploidy, Hymenoptera, sex ratio

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Sex allocation in Hymenoptera has been the focus of much theoretical and empirical works in evolutionary biology (Charnov 1982; Wrensch & Ebbert 1993; Hardy 2002). However, a major problem in the study of sex allocation in Hymenoptera is to determine sex ratio changes during brood development. Sex ratios are usually determined at oviposition (primary sex ratio) and/or the adult stage (secondary sex ratio). The difference between primary and secondary sex ratios may stem from two possible causes. First, it may reflect sex-specific differences in brood mortality during development. Males of Hymenoptera usually develop from haploid eggs and females from diploid eggs. Males being haploid, they could experience higher mortality due to deleterious recessive alleles, as compared to diploid females (Smith & Shaw 1980). Second, in eusocial Hymenoptera, sex-ratio variations during development may also result from a queen–worker conflict over sex ratio (Trivers & Hare 1976). Because of haplodiploidy, workers are more closely related to their sisters than to their brothers. They may therefore maximize their inclusive fitness by raising a female-biased sex investment ratio. By contrast, queens are equally related to their sons and daughters, and are selected to favour an equal investment in both sexes. Therefore, differences between primary and secondary sex ratio may result from workers biasing sex allocation towards their own genetic interest.

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In several ant species, it has been shown that workers perform sex-allocation biasing by killing male larvae (Chapuisat & Keller 1999). Elimination of male brood entails costs, because most or all of the resources invested in rearing males until their elimination are lost. Hence, the earlier in development that workers are able to identify the sex of the brood, the lower will be the costs of male elimination (Nonacs & Carlin 1990). Despite its theoretical importance, the developmental stage at which males are selectively eliminated has received surprisingly little attention. This results mainly from the difficulty in determining the sex of the brood.

Here, we show that flow cytometry, a method for the determination of ploidy level based on the measurement of nuclear-DNA content, can be successfully used to discriminate accurately between haploid and diploid brood in the ant *Linepithema humile*. Cross-species analyses indicate that flow cytometry also allows determination of brood sex in other ant species belonging to different subfamilies, as well as in solitary Hymenoptera.

Adults ($n = 3$ males and $n = 3$ female sexuals) and unknown sex larvae at four different developmental stages (large: 1.3–1.8 mm, $n = 3$; medium: 1.1–1.3 mm, $n = 6$; small: 0.6–1.0 mm, $n = 6$; very small, i.e. immediately after egg hatching: < 0.6 mm, $n = 25$), and eggs (0.32–0.38 mm, $n = 8$) were taken from a large colony of *L. humile* and used for flow cytometry analysis. Individuals (adults, larvae, eggs) were analysed one at a time. They were chopped with a sharp razor blade in a Petri dish to free interphase

nuclei. At this stage, the nuclear-DNA content reflects the ploidy state of the sample. Nuclei were prepared following the procedure of Vindelov *et al*. (1983), which was initially developed for mammalian cells. The nuclei were suspended in 200 µL of solution A (trypsin: 1.5 mg/50 mL buffer solution: 200 mg trisodium citrate.2H_2O, 104.4 mg spermine tetrahydrochloride, 12.1 mg Tris(hydroxymethyl) aminomethane, 200 µL Igepal, 200 mL dH_2O; pH 7.6). After 10 min, 150 µL of a solution B (25 mg trypsin inhibitor, 5 mg ribonuclease A, 50 mL buffer solution) was added. Ten minutes later, 150 mL of an ice-cold solution C (20.8 mg propidium iodide, 58 mg spermine tetrahydrochloride, 50 mL buffer solution) was added. Preparations were kept in darkness at +4 °C for 30–60 min before flow cytometric analysis. Propidium iodide (PI)-stained nuclei from each individual were acquired on a Facscan flow cytometer (Becton Dickinson with air cooled Argon ion laser emitting at 488 nm) and data were analysed by using the software CellQuest®. Lymphocyte nuclei from human peripheral blood were used as standard for the calibration of the flow cytometer. DNA content of 2500 nuclei was analysed from each individual after exclusion of nonnuclear fluorescent debris of tissues and nuclear doublets by pulse-shape analysis, i.e. evaluation of fluorescence area vs. fluorescence width of the incoming signals. Since doublets are bigger than single nuclei, they take longer to pass through the laser beam, resulting in a larger pulse width.

Preparation of PI-stained nuclear suspension resulted in very little artefacts such as fluorescent nonnuclear debris and nuclear aggregates when applied to insect cells. DNA histograms of *L. humile* adults and unknown sex larvae show two major peaks of nuclei frequency (Fig. 1). The first peak corresponds to the distribution of nuclei over G0/G1 (i.e. the ploidy level of the sample with 1C DNA and 2C DNA for males and females, respectively), and the second peak corresponds to the distribution of nuclei over G2/M of the cell cycle stages. Other peaks occurring in the region higher than peak 2 correspond to the distribution of polyploid cells (Fig. 1e, 4C DNA). Small signals appearing in

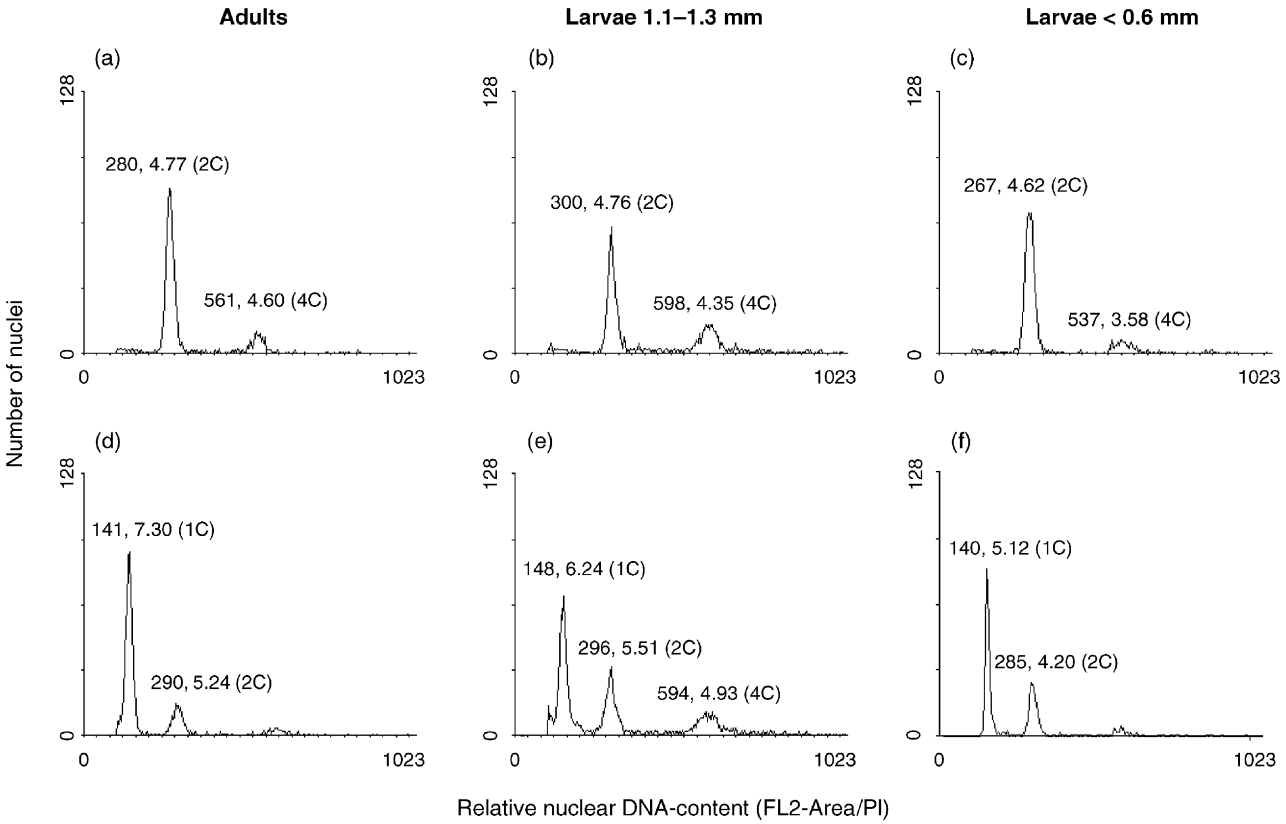

Fig. 1 Flow cytometric DNA histogram of adults (a: female, d: male), and medium (b, e) and small sized (c, f) unknown sex larvae of the Argentine ant *Linepithema humile*. Each histogram shows the nuclear frequency with regard to DNA content for a single individual. Above: diploid-female samples; below: haploid-male samples. The first peak corresponds to ploidy level, the second peak to nuclei with a double DNA content and the third peak to polyploid nuclei. The relative mean value of the nuclear-DNA content (channel number) and the coefficient of variation are reported above each peak.

the region lower than peak 1 result from disrupted nuclei and/or nonspecific staining of other cell constituents.

The amount of DNA in G0/G1 nuclei from adult females is about twice as much as that of nuclei from adult males. Such a difference in DNA content between haploid males and diploid females also occurs for all larval stages. As shown Fig. 1(c and f), ploidy level can be determined accurately, even for larvae < 0.6 mm, i.e. at the very first larval stage. Over all samples analysed, the coefficients of variation for the G0/G1 were relatively low, ranging from 4.2% to 7.3% (mean ± SD: 5.22% ± 1.07; 95% CI: 4.77–5.67; $n = 46$). Within each class of sex (haploid or diploid), the mean fluorescence intensity in G0/G1 nuclei varied slightly according to the developmental stage (haploids: range 141–149, $n = 7$; diploids: range 267–307, $n = 39$). However, the distributions of the relative DNA quantity in both classes did not overlap (haploids: range 100–182; diploids: range 240–358). Thus, the ploidy level of each individual may be assigned without any ambiguity.

Flow cytometric analysis did not allow identification of ploidy level at the egg stage ($n = 8$); indeed, no major peak of nuclei frequency occurred on DNA histograms.

These results show that flow cytometry can be used successfully to distinguish between haploid males and diploid females in the ant *Linepithema humile*. Flow cytometry allows the sex of the brood to be determined accurately, even at the very first larval stage, i.e. immediately after egg hatching. Cross-species analyses reveal that the method is also reliable for sexing the brood in other ant species, as well as in bees and solitary Hymenoptera (Table 1). Especially, data obtained for the honeybee on larvae of known sex confirm the high reliability of the method.

Two nonexclusive explanations may account for the fact that the ploidy level of eggs could not be determined. First, it may stem from the relatively low number of cells in embryos. Second, it may result from nuclei not surviving the staining technique, which partially degrades chromosomal proteins to get high resolution of quantitative staining. Primary sex ratio determination in ants therefore requires using other methods, such as karyotypes, microsatellites or fluorescence *in situ* hybridization (de Menten *et al*. 2003).

The detection of three peaks in some histograms (Fig. 1e) reveals the presence of polyploid cells in *L. humile*. Polyploidy is a widespread phenomenon in plants and animals — especially arthropods (White 1973; Léry *et al*. 1999). It has been mostly explained by two different cell cycle variations: endoreplication and endomitosis (Edgar & Orr-Weaver 2001). Karyotypes analyses have previously revealed polyploidy in other ant species as well (Imai & Yosida 1965; Agosti & Hauschteck-Jungen 1987). Whether polyploidy in ants stems from endoreplication or endomitosis remains currently unknown.

Besides the reliability of the method, a major advantage of flow cytometry is its rapidity. Once the samples have been labelled with the fluorescent dye (about 30 min for 20 individuals), the DNA content of PI-stained nuclei from each sample can be determined within 5–15 s.

The ability to determine sex ratio variations during larval development is of great importance for students of queen–worker conflict in ants, because it allows the assessment of when do workers identify the sex of the brood and bias sex ratio. Precise estimations of brood sex ratio variations may also be of particular interest for people working on solitary Hymenoptera. For example, flow cytometry should facilitate the study of sex-specific differential mortality (Godfray 1994) by determining the developmental stage of the brood at which lethal recessive alleles are expressed in haploid males. Another potential application of flow cytometry is the determination of distinctive genetic characteristics, such as diploidy in males (see *Solenopsis invicta*, Table 1) or triploidy in females.

Overall, our data show that flow cytometry is a good tool for the study of ploidy level and sex brood determination in ants and other Hymenoptera. It represents a rapid and reliable alternative to other techniques (karyotypes, microsatelites markers) used to date to differentiate between males and females in haplodiploid organisms.

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Table 1 Ploidy level determination by flow cytometry in social and solitary Hymenoptera. The mean fluorescence intensity in G0/G1 nuclei (peak channel), the coefficient of variation and the observed ploidy level are reported for each individual. Data are given for 2500 nuclei (adults) or 500 nuclei (larvae) after exclusion of debris and nuclear aggregates by pulse-shape analysis. Samples: adult males, adult females (workers or sexuals) and unknown sex larvae (1–3: larval developmental stage)

Subfamily	Species	Sample	Peak channel G0/G1	CV (%)	Observed ploidy level
Formicinae	*Plagiolepis pygmaea*	Female	397	4.96	2n
		Male	202	6.03	n
		Larva 1*	396	2.56	2n
		Larva 1*	199	5.59	n
	Camponotus lateralis	Female	388	4.45	2n
		Male	192	5.33	n
		Larva 1 or 2†	191	5.02	n
Myrmicinae	*Monomorium pharaonis*	Female	434	4.66	2n
		Male	244	4.60	n
		Larva 1‡	234	5.00	n
		Larva 1‡	469	5.90	2n
	Solenopsis invicta	Female	414	2.56	2n
		Male	207	6.72	n
		Male	408	3.50	2n
		Larva 3§	402	2.53	2n
Ponerinae	*Pachycondyla apicalis*	Female	647	4.52	2n
		Male	321	6.20	n
Apidae	*Apis mellifera*	Female	409	4.50	2n
		Male	202	9.66	n
		Female larva¶	407	5.01	2n
		Male larva¶	201	10.65	n
Eupelminae	*Eupelmus vuilleti*	Female	374	2.41	2n
		Male	196	2.47	n
		Larva 1**	187	7.53	n
Aphidiinae	*Aphidius rhopalosiphi*	Female	347	9.84	2n
		Male	173	8.36	n
Braconinae	*Coeloides bostrichorum*	Female	401	3.54	2n
		Male	200	3.37	n

*Larval length: < 0.43 mm, hairless, i.e. first instar (Passera 1968).
†From worker-laid eggs in queenless colonies. Larval length: 1.5 mm. No data available on larval development in *C. lateralis*. In the slightly larger species *C. aethiops*, larval lengths are 1.2–1.8 mm, 1.8–2.3 mm and 2.3–3.2 mm for the first, second and third instars, respectively (Dartigues 1978).
‡Larval length: 0.4 mm, i.e. first instar (Berndt & Kremer 1986).
§Larval length: 0.4 mm, i.e. third instar (Petralia & Vinson 1979).
¶In the honeybee, males and workers are reared in different-sized cells; it is therefore possible for the experimenter to know the sex of the brood. Data were obtained from larvae < 1 day old.
**From an egg laid by an unmated female about 48 h before analysis (E. Darrouzet, personal communication).

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