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Sperm morphology: A novel way to associate female-males of highly sexual dimorphic fig wasp species

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Abstract
Males of pollinating and some non-pollinating fig wasps are wingless and quite dissimilar to their co-specific females. Due to the accentuated sexual dimorphism, males and females of some fig wasp species were described in different genera. We used morphological sperm features obtained from male seminal vesicles and female spermathecas to associate sexes in three non-pollinating fig wasp species, genus *Idarnes*, that are associated with *Ficus citrifolia* in Brazil. Sperm obtained from each female morph species presented diagnostic features that led to the association with sperm obtained from males. This method can potentially be used to help enlighten taxonomic problems in other wasp species with sexual di- or polymorphism.

Keywords: *Ficus citrifolia*, Moraceae, *Idarnes*, mutualism, sexual dimorphism

1. Introduction

The fig-fig wasp mutualism is regarded as a classic study system in both evolutionary biology and insect-plant ecology. Figs play an important role in tropical ecosystems, as they are involved in a number of ecological interactions with diverse groups of animals (Kjellberg et al., 2005).

Males of pollinating and some non-pollinating fig wasps are wingless and quite dissimilar to their co-specific females. In addition, males of some non-pollinating fig wasp (NPFW) species are polymorphic and have either wingless and winged discrete morphs, or a continuous series from wingless to full winged ones (Bouček, 1993; Cook et al., 1997). Due to the accentuated sexual dimorphism, males and females of some fig wasp species were described in different genera and such mistakes were only corrected years later. The Neotropical non-pollinating genus *Idarnes* is an example of such taxonomic mistake. Wingless males of *Idarnes* were originally described as *Ganosoma* and the winged females as *Tetragonaspis*, and later Fritz Müller realized that both belonged to the same species (Müller, 1886). As this example shows, identifying co-specific sexes in such dimorphic species is not a straightforward task.

In Brazil, it is common to find three sexually dimorphic *Idarnes* morph species in the same fig of *Ficus citrifolia* P. Miller, two belonging to the *carme* group and one to the *flavicollis* group (Pereira and Prado, 2005). Two different methods have been used to link sexually dimorphic sexes in fig wasp species. One of them is to find figs from which females of only one species emerged and associate males to them (Müller, 1886). However, large samples are necessary to find such particular situation. Another method is to use molecular markers to identify specimens (Molbo et al., 2002). This method is more expensive and requires a genetic laboratory and development of specific primers.

In the present study, we explore the use of morphological sperm features obtained from male seminal vesicles and female spermathecas as an alternative method to associate sexes, exemplified by the three referred species of *Idarnes*, from the *flavicollis* and *carme* groups. We classified females and males into morph species according to their morphological characters and, then we associated female and male morphs due to morphological sperm

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similarities. There are potentially many undescribed fig wasp species in the Neotropics, as well as in other regions of the world (J.Y. Rasplus, personal communication), and this method may help to pair sexes in these new species.

2. Materials and Methods

Study species

Ficus citrifolia (subgenus *Urostigma*, section *Americana*) is a monoecious hemi-epiphytic tree that frequently develops within disturbed areas (personal observation). In Brazil, *F. citrifolia* is pollinated by a *Pegoscapus* species (*P. near tonduzii* Grandi) and associated with other fourteen non-pollinating chalcid wasp species (Pereira et al., 2000). Most of the non-pollinating species on *F. citrifolia* belongs to the genus *Idarnes*. There are three species with wingless males, two of them belonging to the *carme* group (*Idarnes* sp. 1 and sp. 2) and the third one to the *flavicollis* group (sp. 3), as well as two species with winged males belonging to the *incerta* group.

Wasp sampling and sperm characterization

We sampled about 40 figs shortly before the wasp emergence phase from one tree in the surroundings of the campus at Universidade Estadual de Campinas (22°54'S; 47°03'W), in July 1999. Some figs were cut open to obtain recently emerged males. Other figs were placed in sealed plastic flasks to let wasps mate and emerge naturally from the fig.

About three to 10 females and males of each species were dissected in a drop of a phosphate buffer solution [PBS, pH 7.2, Pearse (1953)] to remove spermathecas and seminal vesicles, respectively. Then, females were grouped into morph species, according to their morphological characters, and male heads and thoraces were kept in 70% alcohol for further morphological description. Spermathecas and seminal vesicles were transferred to histological slides with a drop of PBS. Then, spermathecas and seminal vesicles were cracked open to release sperm, which was fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate for 5 min and washed in running water. After drying at room temperature, slides were observed with a phase-contrast photomicroscope (Olympus BX60).

To measure nuclei, slides were stained for 15 min with 0.2 mg/ml 4,6-diamino-2-phenylindole (DAPI) in PBS, washed, and mounted with Vectrashield (Vector Laboratories). Then, these slides were examined with an epifluorescence microscope (Olympus, BX60), equipped with a BP360-370 nm excitation filter.

We observed the sperm morphology and measured lengths of both sperm and nuclei. Sample sizes for sperm analyses are shown in Table 1.

Table 1. Number of wasps and sperm (in brackets) used to measure length of sperm and nuclei.

Sex	Morphospecies	Sperm length	Nucleus length
Female	sp. 1	3 (3)	3 (19)
	sp. 2	4 (10)	3 (41)
	sp. 3	6 (26)	3 (26)
Male	Morph 1	2 (10)	2 (18)
	Morph 2	4 (34)	1 (14)
	Morph 3	4 (42)	1 (8)
	Morph 4	2 (13)	1 (6)

Male characterization and sex association

We labeled each sampled male and grouped them into morphs, according to their head morphologies. Then, we associated male morphs to female morph species by comparing the morphology and size of sperm that was sampled from seminal vesicles and spermathecas.

We used a large fig collection that had been previously sampled from fifteen fig trees (~600 figs) in the study site to validate the method. From these figs, we could select ten figs where only one female morph species of *Idarnes* had occurred. Assuming that all males and females present in these figs belonged to the same species, we verified whether male-female pairs in such figs corresponded to the association determined by comparing sperm.

3. Results

We recognized four male morphs based on their head morphology. The head of morph 1 presented an evident longitudinal suture centered on the cephalic capsule (Fig. 1A), which was a little longer than wide. Heads were quadrangular in morphs 2 and 3, but morph 3 presented a conspicuous process on the clypeus region (Figs. 1B and C). Morph 4 was smaller than the other morphs and had a sub-trapezoidal head (Fig. 1D).

We associated male morph 1 with female sp. 1, morph 2 with female sp. 2 and both morphs 3 and 4 with female sp. 3 (Figs. 2 and 3), according to both morphological and morphometric similarities found between sperm from seminal vesicles and spermathecas.

Female sp. 1 and male morph 1 showed, in general, the longest sperm with the smallest nucleus (Fig. 3). Flagella have a helicoidal appearance along the entire length, and DAPI-stained fluorescence micrographs revealed lance-like nuclei (Figs. 2A and D).

Female sp. 2 and male morph 2 have the shortest sperm with less wavy flagella. DAPI coloration revealed wavy nuclei in the anterior part (Figs. 2B and E). In this species, some sperm from the seminal vesicles had nuclei with

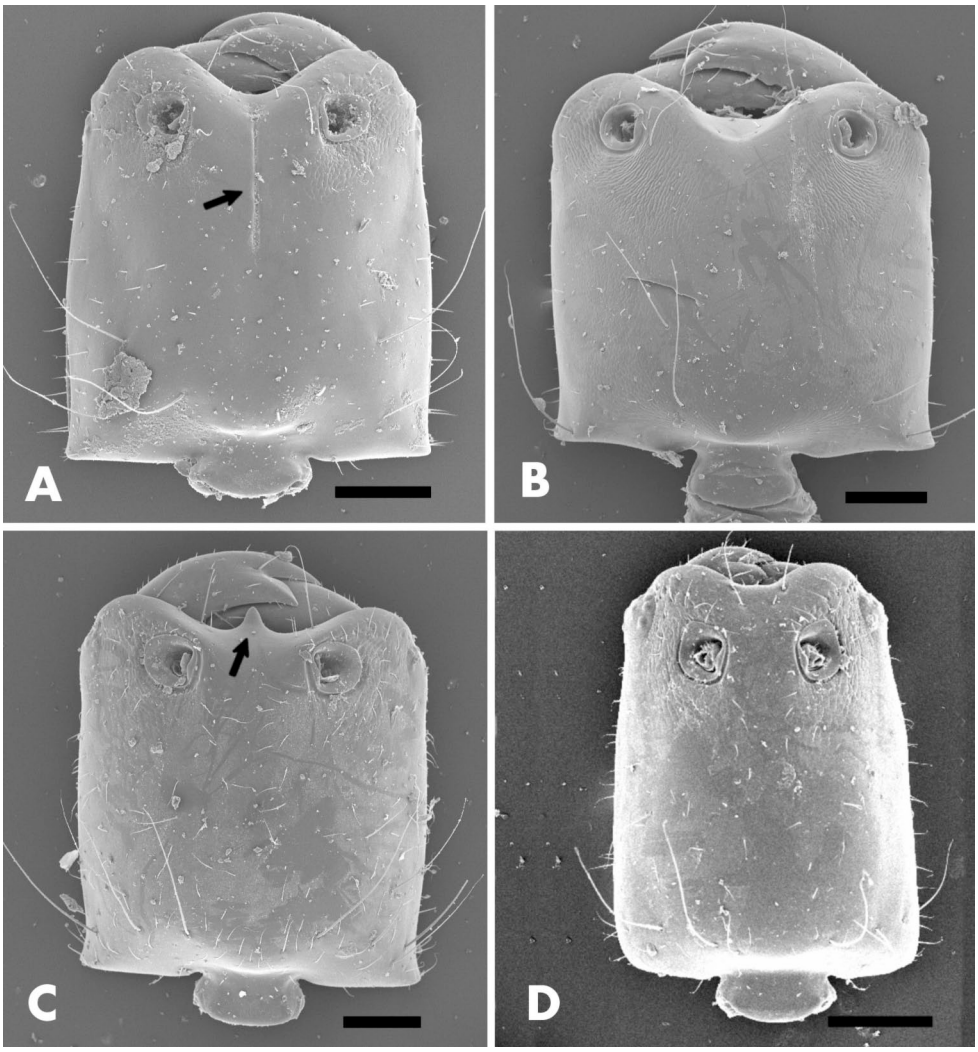

Figure 1. Scanning electron micrographs of heads of *Idarnes* males. A – morph 1, note the longitudinal suture (arrow); B – morph 2; C – morph 3, note the process on the clypeus region (arrow); D – morph 4. Scale bars = 100 μm.

uncondensed chromatin (Fig. 2E). These nuclei were frequently unattached from the flagella, thus suggesting that they might not be viable.

Medium-length sperm was found in female sp. 3 and males morph 3 and morph 4. Light micrographs revealed wavy flagella along the entire length in these morph species (Figs. 2C and 3). Nuclei in this group were similar in size to those of sp. 2, but they were slightly wavy and diameters were more regular along the entire length (Fig. 2F).

Data from all ten figs, from which only female of only one *Idarnes* species had occurred, validated the female-male association based on the sperm morphology. We found only morph 1 males in the figs in which there were only females of sp. 1. We found only morph 2 males in the figs in which there were only females of sp. 2. Out of six figs with only females of sp. 3, morph 3 males were found in two, morph 4 males were found in two, and morph 3 and 4 males were found in the other two figs.

4. Discussion

The correct female-male association among fig wasp species that present accentuated sexual dimorphism could be achieved with the method described in the present study. Data obtained from figs in which only one *Idarnes* female morph species had occurred validated links that were established by comparing sperm. Advantages of the sperm comparison method are: (a) the use of standard laboratory equipment, (b) lower costs than those of molecular techniques, and (c) the limited number of samples needed. Moreover, the present methodology led to an unequivocal interpretation of male dimorphism in *Idarnes* sp. 3.

Male di- and polymorphism is reported in *Idarnes* (Gordh, 1975) as well as in other fig wasp genera. For recent publications on male polymorphism see Jousselin et al. (2004), Cook and Bean (2006), Moore et al. (2004) and references cited by them. Distinguish between intraspecific

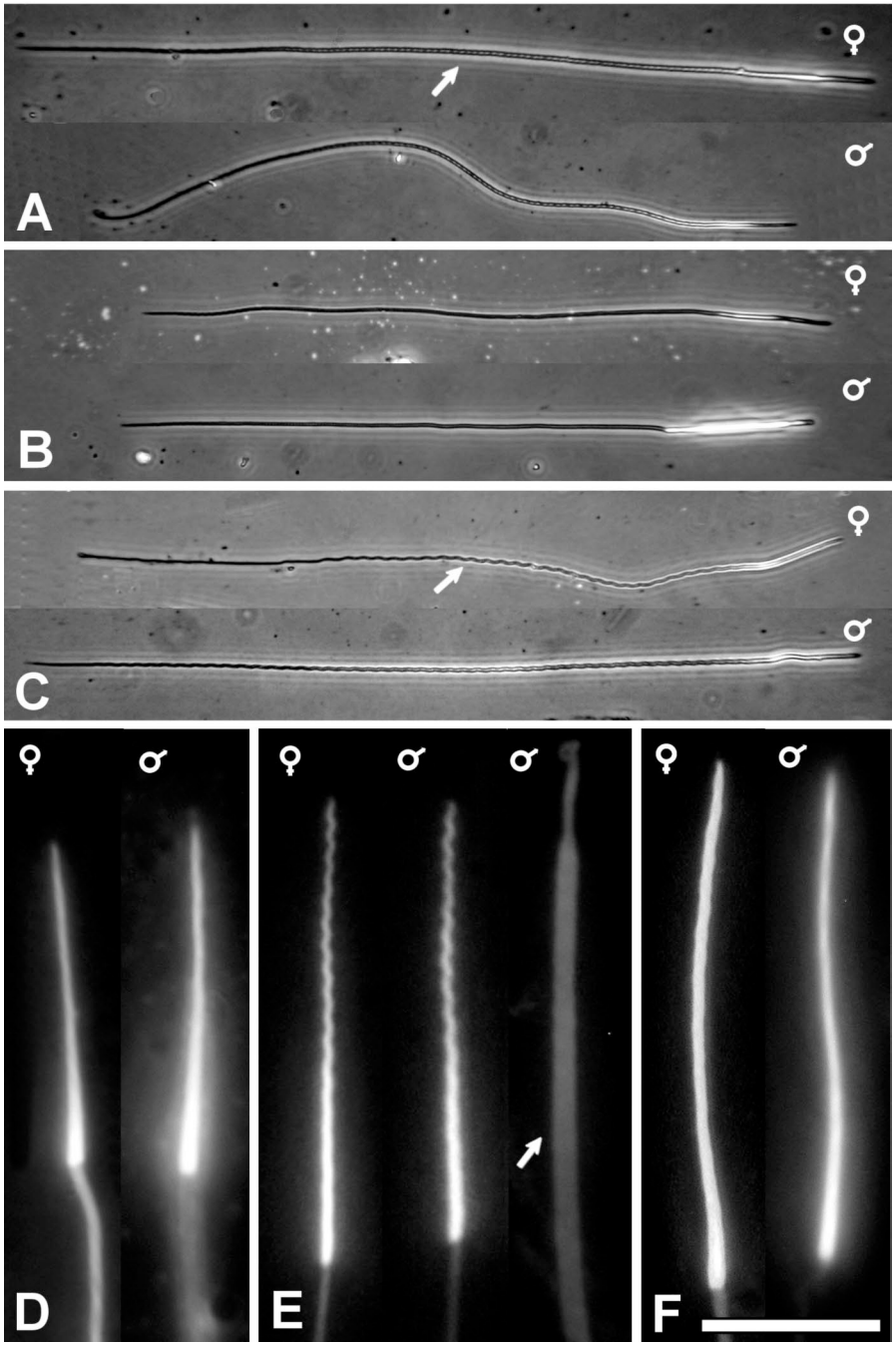

Figure 2. Micrographs of sperm sampled from spermathecas (♀) and seminal vesicles (♂) of *Idarnes* spp. wasps. A–C: sperm of sp. 1 (morph 1), sp. 2 (morph 2) and sp. 3 (morphs 3–4), respectively. Note the appearance of flagella (arrows). D–F: sperm nuclei in sp. 1, sp. 2 and sp. 3, respectively. Note the uncondensed acrosome in sp. 2 (arrow). Scale bars = 50 μm (A–C) and = 10 μm (D–F).

polymorphs and interspecific variation may not be ever a straightforward task. For that reason, grouping polymorphic males by sperm morphology makes it easy to find diagnostic external morphological features to identify male species. In our study this method showed that head morphology of males have enough diagnostic features to identify the four morphs in the study *Idarnes* species.

Association of males and females in di-and polymorphic species by using only external wasp morphology requires both good taxonomic skills and large sample sizes, so that sexes can unequivocally be matched, even in figs where potentially bewildering species do not occur simultaneously. The observation of mating might be another way to associate sexes in such species, but this is time consuming and large samples are possibly required.

Our results suggest that sperm morphology is highly variable among NPFW species. Quantitative and qualitative variations in sperm characters were found between closely

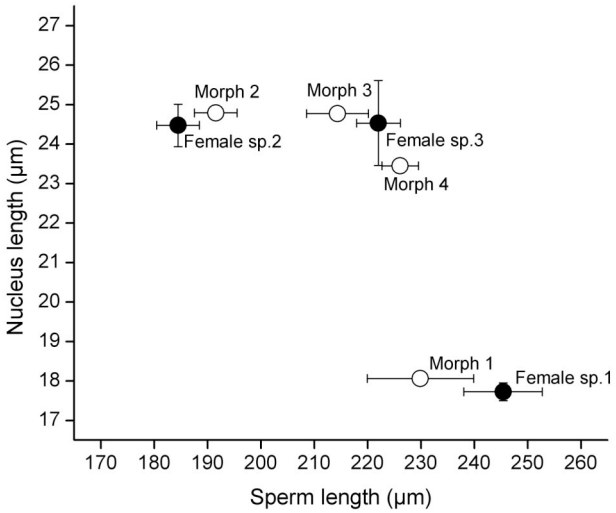

Figure 3. Total sperm length and sperm nucleus length of *Idarnes* female morphospecies and male morphs. Circles are means per wasps and bars are standard errors of means. Sample sizes are shown in Table 1.

related *Idarnes* species of the *carme* group. Our results suggest that sperm morphology is a potential diagnostic feature, which can be used to help enlighten taxonomic problems in other wasp species with sexual di-or polymorphism.

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