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Same but different: Larval development and gall-inducing process of a non-pollinating fig wasp compared to that of pollinating fig-wasps

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ABSTRACT

The receptacles of fig trees (*Ficus* spp.) can harbor a highly diversified and complex community of chalcid wasps. Functional groups of fig wasps (e.g. gallers, cleptoparasites and parasitoids) oviposit into the fig at different developmental stages, reflecting different feeding regimes for these insect larvae. There are few direct data available on larval feeding regimes and access to resources. We studied the gall induction and larval feeding strategy of an *Idarnes* (group *flavicollis*) species, a non-pollinating fig wasp (NPFW) associated to *Ficus citrifolia* P. Miller in Brazil. This *Idarnes* species shares with the pollinator characteristics such as time of oviposition, ovipositor insertion through flower and location of the egg inside plant ovaries. Nevertheless, we show that the gall induction differs considerably from that of the pollinating species. This *Idarnes* species relies on the induction of nucellus cell proliferation for gall formation and as the main larval resource. This strategy enables it to develop in both pollinated and unpollinated figs. The large differences between this NPFW and other fig wasps in how ovules are galled suggest that there are different ways to be a galler. A functional analysis of NPFW community structure may require descriptions of the histological processes associated with larval development.

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1. Introduction

In a synthesis on the evolution of ecological specialization, Forister et al. (2012) highlighted "the importance of interactions for understanding specialization at all levels of biological organization". The ideas were mainly drawn from systems including plants, herbivorous insects and their enemies. Open questions for future research directions included: "how does the network structure of species interactions in a community affect the distribution and evolution of specialists and generalists?" and "what are the community consequences of ecological specialization?"

Describing network structure requires a proper understanding of the biology of the individual species involved and understanding the determinants of network structure requires comparing series of similar networks. Figs and the communities of chalcid wasps colonizing figs constitute a remarkable system to investigate the determinants of community structure and the evolution of specialization. Indeed, intricate communities of chalcid wasps are harbored inside the receptacles of fig trees (*Ficus* spp.) – a genus of Moraceae with more than 700 species (Berg, 1989). Despite the constraints imposed by the morphology of the fig inflorescences and the limited number of accessible plant ovaries for wasp development, these communities can be composed of up to 30 species of fig wasps (Hawkins and Compton, 1992; Cook and Rasplus, 2003). The most singular group of fig wasps is represented by the mutualistic pollinating species. Females pollinating fig wasps enter the receptive urn-shaped *Ficus* inflorescences (hereafter referred to as figs) through a bract-lined entrance (the ostiole). They then lay eggs individually into some of the uniovulate flower ovaries (Galil and Eisikowitch, 1968) whilst simultaneously spreading the pollen they carry from their natal tree onto the stigmatic surface of the flowers (Jousselin et al., 2001, 2003). As a single egg is laid per oviposited flower, one wasp develops at the expenses of a potential seed for the plant.

Along with the pollinators, non-pollinating fig wasps (NPFW) compose most of the diversity of the fig wasp community. A majority of these species oviposit from outside of the fig and represent no benefit for the plant. It is well established that different components

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of the NPFW community oviposit at different developmental stages of the fig. Thus, feeding regime of NPFWs has been inferred from their oviposition time (Hawkins and Compton, 1992; Kerdelhué and Rasplus, 1996; Elias et al., 2008; Wang and Zheng, 2008). Moreover, it is quite intuitive to define functional groups of fig wasps according to the colonization time: 1) gallers, arriving before or during flower receptivity, 2) cleptoparasites of the gallers, arriving after fig receptivity but before fig ripening during what is called the interfloral phase of the fig, and 3) parasitoid wasps, arriving later in the interfloral phase. However, some variation for both colonization time within functional groups and feeding regime of given species has been reported (Pereira et al., 2007; Elias et al., 2008).

Further very little direct data are available on larval feeding regimes and on how the wasps access resources. Indeed, it is not trivial to elucidate feeding regimes in fig wasps, and additional information than colonization time is required. Strategies of female oviposition can be associated to how plant tissue is modified and exploited by the larvae of fig wasps (Jansen-González et al., 2012; Galil et al., 1970; Pereira et al., 2007; Elias et al., 2012). Jansen-González et al. (2012) showed that gall development in an active pollinating fig wasp, *Pegoscapus* n. sp., in *Ficus citrifolia* is partly dependent on plant embryogenesis. They also showed that the main tissue on which the larvae feed is endosperm, derived from embryo sac fertilization in the flower confirming previous observations on actively pollinating wasps (wasps presenting pollen pockets and a behavior to load and deposit pollen) (Galil and Eisikowitch, 1969; Ramírez, 1969). Similarly, in *Ficus carica* L., the pollinator larva feeds on the endosperm (Leclerc du Sablon, 1908). However in that case the initiation of endosperm development is most often parthenogenetic and does not depend on the double fertilization observed in actively pollinated *Ficus* species as the wasps colonizing the main crop of male figs emerge from figs containing little or no pollen (Neeman and Galil, 1978). Frequent development of pollinator larvae in unfertilized ovules could be generalized in passively pollinated figs (Jousselin et al., 2004). On the other hand, the parasitic species *Sycophaga sycomori* L., a NPFW galler in *Ficus sycomorus* L. oviposits from inside the fig and larvae feed on hypertrophied nucellus, a tissue independent from pollination and fertilization (Galil et al., 1970).

As a further complication, oviposition and larval feeding regimes can change according to circumstances. A cleptoparasitic wasp *Idarnes* group *carme* (on *F. citrifolia*) has been shown to use intact seeds as alternative oviposition sites when galls become scarce (Pereira et al., 2007). Studying in detail the larval biology and gall-inducing process of fig wasps, especially within lineages presenting contrasted feeding regimes, can shed light on how different feeding niches within the community are filled, how parasitic strategies evolved inside this community and how mutualism may persist despite the presence of parasitic forms.

Here we studied the gall-inducing process and larval feeding strategy of a NPFW galler associated with *F. citrifolia* in Brazil. The study species *Idarnes sp.* (group *flavicollis*) belongs to the Sycophaginae within which feeding regimes have shifted multiple times (Cruaud et al., 2011). It colonizes figs at the same time as pollinators. Even though the *Idarnes* group *flavicollis* female oviposits from outside the fig, its ovipositor is inserted in the plant ovary through the flower style, as is the case for the pollinating wasps (Elias et al., 2012). This suggests that the oviposition behavior of this *Idarnes* species and the pollinating species have in some aspects emerged by convergent evolution. Experimental data has showed that *Idarnes* group *flavicollis* can successfully gall flowers containing unfertilized embryo sacs (Elias et al., 2012). We investigated how resources are modified and exploited by the larvae, and whether gall induction and larval feeding strategies change in pollinated and unpollinated fig flowers.

2. Material and methods

2.1. Study species

F. citrifolia (subgenus *Urostigma*, section *Americana*) is a monoecious fig tree, and it is actively pollinated by an undescribed *Pegoscapus* species in São Paulo state (J.Y. Rasplus, pers. com.). *Idarnes* is a monophyletic group of NPFW (Cruaud et al., 2011). Bouček divided *Idarnes* in three morphological species groups: *incerta*, *flavicollis* and *carme*. In São Paulo state, Brazil, mainly one undescribed species belonging to the group *flavicollis* (morphospecies 3) is associated with *F. citrifolia*, and a second one is present in very low abundance. The two species are marginally larger than the pollinating wasp and produce marginally larger galls. For simplicity hereafter we refer to this first species as *Idarnes*.

2.2. Development of wasps in pollinated and unpollinated flowers

We studied *F. citrifolia* trees growing naturally on the campus of São Paulo University, Ribeirão Preto, Brazil (21°10′S; 47°48′W), between September 2010 and October 2011. We studied five cohorts of wasps, each from a different tree. We studied the development of *Idarnes* larvae in pollinated figs of three trees and unpollinated figs of two trees.

For each tree, we isolated approximately 150 figs before receptivity from 10 branches with white fabric bags to prevent natural wasp infestation. When the figs became receptive, we introduced *Idarnes* females into each bag (four wasps per fig). Receptivity was determined by the arrival of pollinating wasps and *Idarnes* females to the surrounding untreated figs. The wasps were allowed to oviposit in the bagged figs and removed after 24 h. The unpollinated treatment consisted of introducing only *Idarnes*. The pollinated treatment consisted of injecting a 2% sucrose solution containing fresh pollen of *F. citrifolia* through the ostiole. This sucrose concentration was determined based on pollen germination tests with sucrose percentages ranging from 2% to 40% (Kearns and Inouye, 1993) and has previously been successfully used in figs (Neeman and Galil, 1978). The sucrose + pollen solution was injected by syringe a few minutes before the introduction of the wasps.

We collected wasps and pollen for the experimental introductions the same day from other nearby *F. citrifolia* trees with figs at the wasp emergence phase. The development of each cohort was synchronized by performing all introductions for a particular tree on the same day.

The synchronized introductions allowed us to follow larval development by collecting the experimental figs at different times after introduction of the wasps. To do this, we collected four to five figs per tree every two days from the introduction date. After collection, the figs were fixed for 24 h in FAA 50 (formalin: acetic acid: alcohol 50%; Johansen, 1940) and then transferred to a solution of 70% ethanol. Each fig was cut open under 40× magnification stereomicroscope to sample 20 galled ovaries. Oviposited ovaries were recognized by the scar made through the style by the female ovipositor. We sampled figs until wasp pupae were detected. We measured body length and maximum width in lateral view for each larva under stereo-microscope, using IM50 Leica ™ software.

Due to the lack of evident diagnostic structures related to instar changes (e.g. remains of cephalic capsule), which is a common limitation in Microhymenoptera (Clausen, 1962; Stehr, 1987) and the absence of evident morphological differentiation between instars, larval stages were defined based on size changes throughout larval growth and events related to these changes.

For the micro-structural study (hereafter referred to as the histological study), we sub-sampled a group of 10–15 galled

flowers and normally developing seeds per fig at four-day intervals from the initial day of wasp introduction. Each group of material was processed according to standard dehydration and softening protocols, embedded in Leica Historesin® (Gerrits, 1991) and then sectioned with a Leica RM 2245 microtome into 5–6 μm sections. Serial sections were stained with toluidine blue 0.05%, pH 4.4 (O'Brian et al., 1964) and slide mounted. Illustrations were taken using a digital camera coupled to a Leica DM 4500 microscope. All histological slides and fig wasp samples are in possession of R.A.S. Pereira (Plant Ecology Laboratory, FFCLRP/USP) as voucher material.

3. Results

We observed three larval instars in *Idarnes*, as determined by changes in larval growth (Fig. S1 of electronic supplementary material) and morphology (e.g. presence of chitinous mouthparts). However, the detection of instar changes was not clear for all cohorts due to the small changes in size among instars in Microhymenoptera (Harvey et al., 1999, 2004; Damiens et al., 2001).

One egg was laid per flower, near the flower stylar canal entry, between the inner integument of the ovule and the nucellus (Fig. 1a–d). The plant ovary underwent considerable changes post-oviposition (Fig. 1a–d). Overall, when compared with normal seeds under development, oviposited ovaries presented volume increase in cells of the nucellus, integument and the endothelium (Fig. 1).

First (beginning approx. 6–15 days after oviposition) and second instars (beginning approx. 15–18 days after oviposition) remained where the egg was laid and grew rapidly. By these stages of development, distinguishing the fertilization status in oviposited flowers was difficult. We did not find plant zygote or endosperm in sections of oviposited flowers of both the pollinated and unpollinated treatments (Fig. 2a–d). Nevertheless, a vestige of embryo suspensor was evident in oviposited flowers in the pollinated treatment (Fig. 2d). Nucellus cells continued to increase in volume, with those around the larva showing a bigger distortion (Fig. 2b and d). Compared to sections of normal seeds of the same age (Fig. 2e and f), the oviposited ovules under the pollinated treatment showed hypertrophied cells inside the embryo sac cavity where endosperm and plant embryo should develop.

During the third larval stage (beginning approx. 20–24 days after oviposition) the larva moved to the micropilar region of the ovule (Fig. 3a–d). All former nucellus, endosperm or embryo cells increased in volume and undergone rapid, disorganized division, as evidenced by the presence of several nuclei in each cell (Fig. 3b and d). Cytoplasm of these hypertrophied cells was dense, due probably to accumulation of storage material. Normal seeds at the same developmental stage presented plant embryo at globular stage and endosperm before the process of cellularization, with no signs of abnormal cell growth (Fig. 3e and f).

4. Discussion

Idarnes shares with the pollinating species the colonization time (fig receptive phase) and the oviposition mode (ovipositor inserted via the styles and eggs laid between the inner integument and the nucellus). However, the way *Idarnes* induces and exploits plant resources is quite different.

Our results demonstrate that gall induction by *Idarnes* is similar in pollinated and unpollinated flowers. In comparison with gall induction by the pollinating species (Jansen-González et al., 2012; Leclerc du Sablon, 1908), *Idarnes* is clearly more aggressive. The induction of galls by *Idarnes* involves the early modification of all cells of the plant's ovule, especially those of the nucellus and integuments. In pollinated flowers neither plant embryo nor endosperm develop. This suggests that these organisms de-differentiate in the gall induction process. These events suggest the *Idarnes* wasps are efficient gall makers, irrespective of whether flowers contained fertilized or unfertilized embryo sacs.

We did not elucidate whether the adult female or the larva is responsible for gall induction. Substances injected by female pollinating wasps during oviposition are apparently responsible for early gall induction (Leclerc du Sablon, 1908; Jansen-González et al., 2012). Nevertheless, it is unknown whether such wasp secretion is responsible for further gall development. Our results suggest that *Idarnes* larvae can be involved in the gall-inducing process. Indeed, once the larva has entered its first and second stages, nucellus around the larva becomes hypertrophied suggesting that secretions from the larva could be involved. A further step forward in the study of gall induction would be to separate maternal and larval contributions to gall induction and development.

Although it is still uncertain why *Idarnes* females present the same mode of oviposition as pollinating wasps, this behavior/mechanism could result in decreased ability of the fig tree to evolve defenses against the NPFW. As all oviposition steps performed before the larvae hatch are similar for the pollinating and *Idarnes* wasps, any plant defense that excludes early stages of *Idarnes* larvae development would probably also exclude pollinator larvae. The strategy adopted by the galler *S. sycomori*, that oviposits internally in the fig is quite different (Galil et al., 1970). In the *Sycophaga* the ovipositor is introduced at the base of the style and not within the style, and the egg is laid in the embryo sac and not in the same position as a pollinator egg. It has to be noted that while the oviposition technique and egg deposition location differ between *Sycophaga* and *Idarnes*, they both belong to the Sycophaginae: generalizations may be misleading. Early cleptoparasites (e.g. *Philotrypesis* spp.), on the other hand, insert their ovipositors through the flower pedicel, even though they colonize the figs almost contemporarily to the pollinators (Joseph, 1958; Compton et al., 2009).

Another selective factor that may explain the mode of oviposition in *Idarnes* is the avoidance of competition with conspecifics and pollinators. The insertion of the ovipositor through the style would help to assess if the flower is occupied in the first place. Then, if the flower is unoccupied, oviposition can follow and byproducts of the process such as damage to stylar tissues and secretion of substances by the female could signal ovule occupancy to subsequent females. Such mechanisms of competition detection may help understand why only a negligible fraction of ovules receive two pollinator eggs as has been quantified for *Kradibia tentacullaris* (Ghana et al., 2012) but seems to be general for other pollinating wasp species (e.g. Jansen-González et al., 2012). Also, as the cost of ovipositing from outside seems to be high for fig wasps in terms of predation risk (Compton and Robertson, 1988) and time expended to access the resource (*i.e.* sites to lay eggs), the ability to detect and avoid occupied flowers may have a positive effect on the females' fitness.

The use of nucellus as main resource for the larvae may be widespread among Sycophaginae species that gall fig ovaries. Larvae of *S. sycomori* also feed on transformed nucellus (Galil et al., 1970). Relying on nucellus gives gallers the ability to develop in flowers with unfertilized embryo sac, as nucellus is already formed at fig receptivity. Moreover, *Idarnes* and *S. sycomori* are able to prevent abortion of unpollinated figs. It is widely reported that gall-inducing arthropods have high concentration of substances such as cytokinins that enable the formation of galls and induce the production of endogenous phytoregulators such as auxins (Elzen, 1983; Dorchin et al., 2009). For the studied *Idarnes* the probability of fig abortion decreased with the number of galls (data not

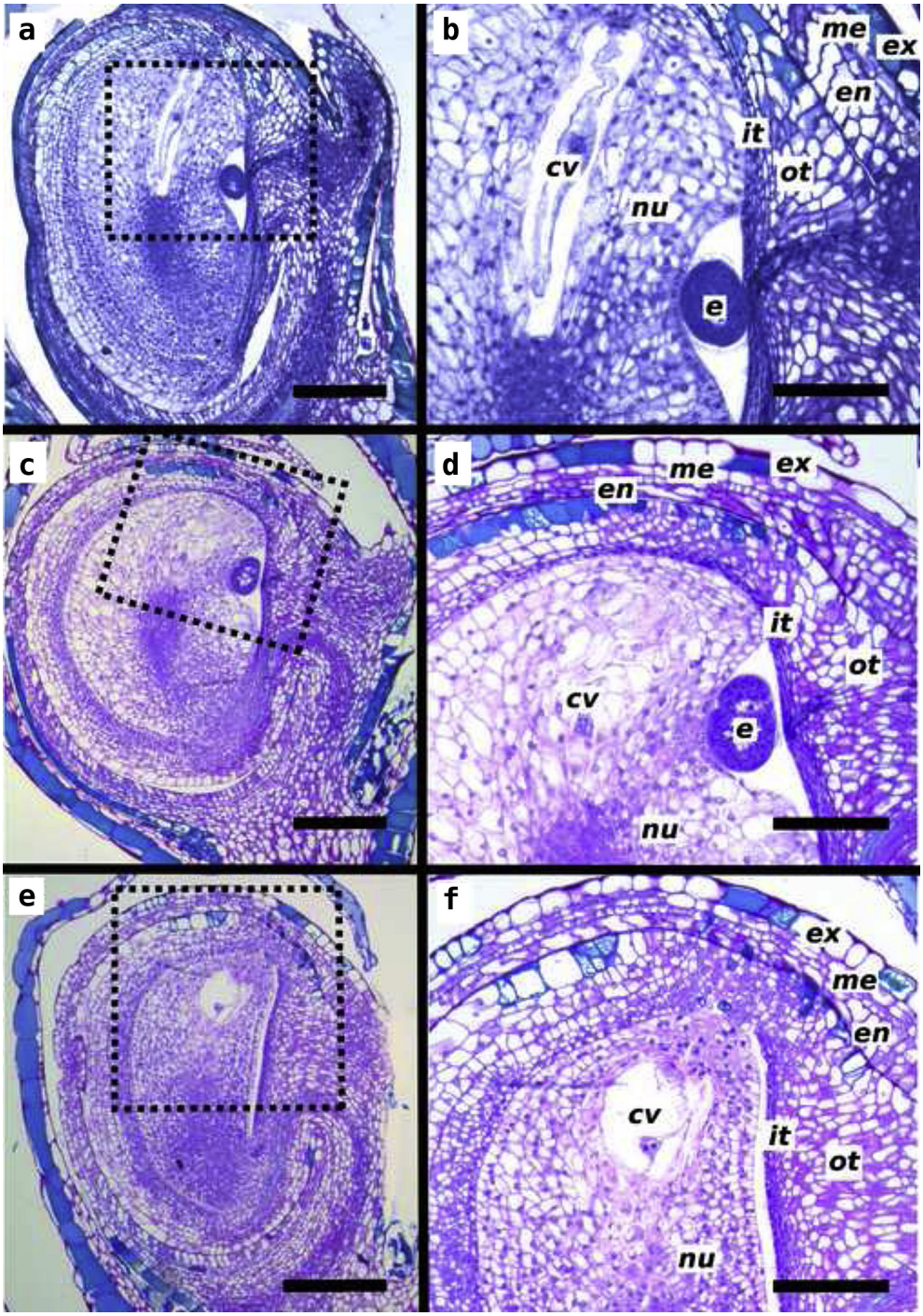

Fig. 1. Longitudinal sections of galls induced by *Idarnes* and normal seeds of *Ficus citrifolia* at early stages of development. (a) general view of a galled flower in the unpollinated treatment; (b) detail of (a) showing the egg located between the nucellus and the inner integument; (c) general view of a galled flower in the pollinated treatment; (d) detail of (c) showing location of the egg; (e) general view of normal seed at the same age; (f) detail of normal seed. *cv* = embryonic cavity. *e* = egg. *en* = endocarp. *ex* = exocarp. *it* = inner integument. *me* = mesoderm. *nu* = nucellus. *ot* = outer integument. Scale bars: (a,c,e) 0.2 mm; (b,d,f) 0.1 mm.

shown), suggesting that a cumulative effect may occur in the infested figs.

Like the larvae of *Idarnes* and *S. sycomori*, larvae of passively pollinating fig wasp have more chances to develop in unpollinated flowers (Jousselin et al., 2004). Nevertheless, in the sole species of passive pollinator for which histological data on the galling process is available (Leclerc du Sablon, 1908), the pollinator larva induces the parthenogenetic development of the endosperm on which it will feed: it does not gall the nucellus as the two gallers, *Idarnes* and *Sycophaga* do (Galil et al., 1970). We may speculate that feeding on the endosperm will only be found in fig pollinating wasps and not in NPFW that gall flowers. If this is true then feeding on the endosperm may have been a pre-adaptation to becoming active pollinators or it may be a trace of the active pollination ancestry of all pollinating wasps suggested by the latest phylogeny of fig pollinating (Cruaud et al., 2012). Interestingly, many actively

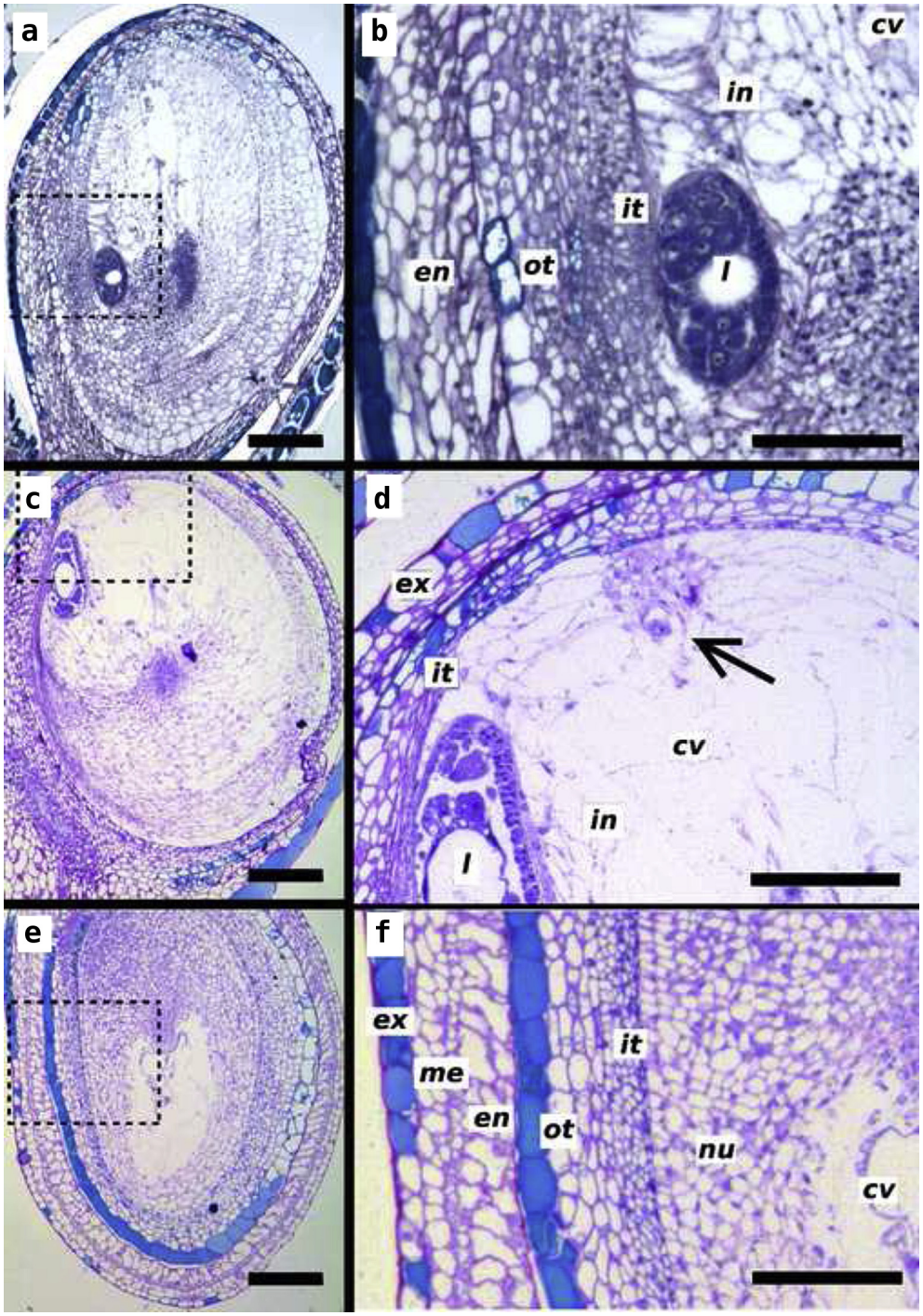

Fig. 2. Longitudinal sections of galls induced by *Idarnes* containing second stage larva and of normal seed in *Ficus citrifolia*. a) general view of galled flower in the unpollinated treatment; (b) detail showing the larvae surrounded by modified nucellus cells; (c) general view of galled flower in the pollinated treatment; (d) detail of (c) showing larva, hypertrophied nucellus and plant embryo cavity, the arrow points to the plant embryo suspensor; (e) general view of normal seed at the same age; (f) detail of normal seed on (e). *cv* = embryonic cavity. *en* = endocarp. *ex* = exocarp. *in* = hypertrophied nucellus. *it* = inner integument. *l* = larvae. *me* = mesocarp. *nu* = nucellus. *ot* = outer integument. Scale bars: (a,c,e) 0.2 mm; (b,d,f) 0.1 mm.

pollinating fig wasps have retained some capacity to develop in unpollinated figs, often with associated costs involved (Jandér and Herre, 2010).

Even though *Idarnes* can develop in unpollinated figs, *i.e.* independently of the pollinating wasp species, its offspring rely on the pollinating species to exit the figs. *Idarnes* males and other common NPFW lack the behavior of chewing the exit hole through the wall of the fig (Elias et al., 2012). Thus, *Idarnes* can escape the fig only when co-occurring with pollinating offspring. The dependence on pollinator males could correlate with supposed behaviors such as the avoidance of oviposition in occupied flowers and the strategy of spreading the offspring in different figs (*i.e.* several figs – small brood size per fig). Such dependence on pollinator population, which is frequent in NPFW associated with other *Ficus* species (e.g. Suleman et al., 2012) could constrain parasitism and favor long-term pollinator survival.

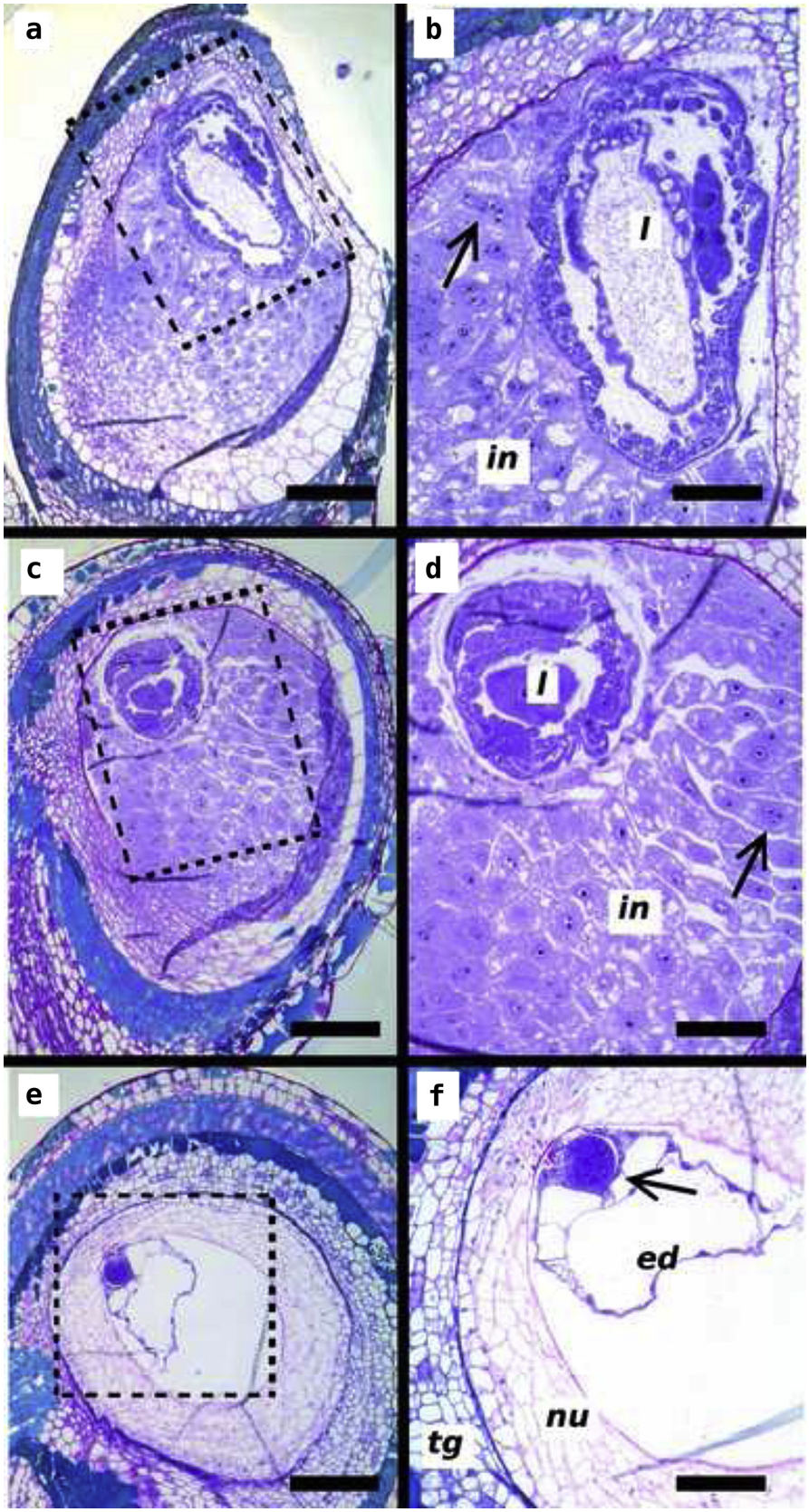

Fig. 3. Longitudinal sections of galls induced by *Idarnes* containing third stage larvae and of normal seed in *Ficus citrifolia*. a) general view of galled flower in the unpollinated treatment; (b) detail showing the larvae at the micropilar region surrounded by modified, dense nucellus cells, arrow points a multinucleated cell; (c) general view of galled flower in the pollinated treatment; (d) detail of (c) showing larva and hypertrophied nucellus, arrow points a multinucleated cell; (e) general view of normal seed at the same age; (f) detail of normal seed with plant embryo at the globular stage (arrow) and endosperm. *ed* = endosperm. *in* = hypertrophied nucellus. *l* = larvae. *nu* = nucellus. *tg* = teguments. Scale bars: (a,c,e) 0.2 mm; (b,d,f) 0.1 mm.

5. Conclusions

We report at a histological level the process of gall development as induced by an *Idarnes* group *flavicollis* species. This species induces cellular proliferation within the nucellus: gall induction is independent of plant fertilization. Despite an abundant literature on NPFWs, this is only the second histological description of how a NPFW wasp galls a fig ovule. The large differences in how ovules are galled, and the differences comparatively to the pollinators, suggest that there are probably many different ways to be a galler. A functional analysis of NPFW community structure may require descriptions of the histological processes associated with larvae development.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.actao.2013.07.003.

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