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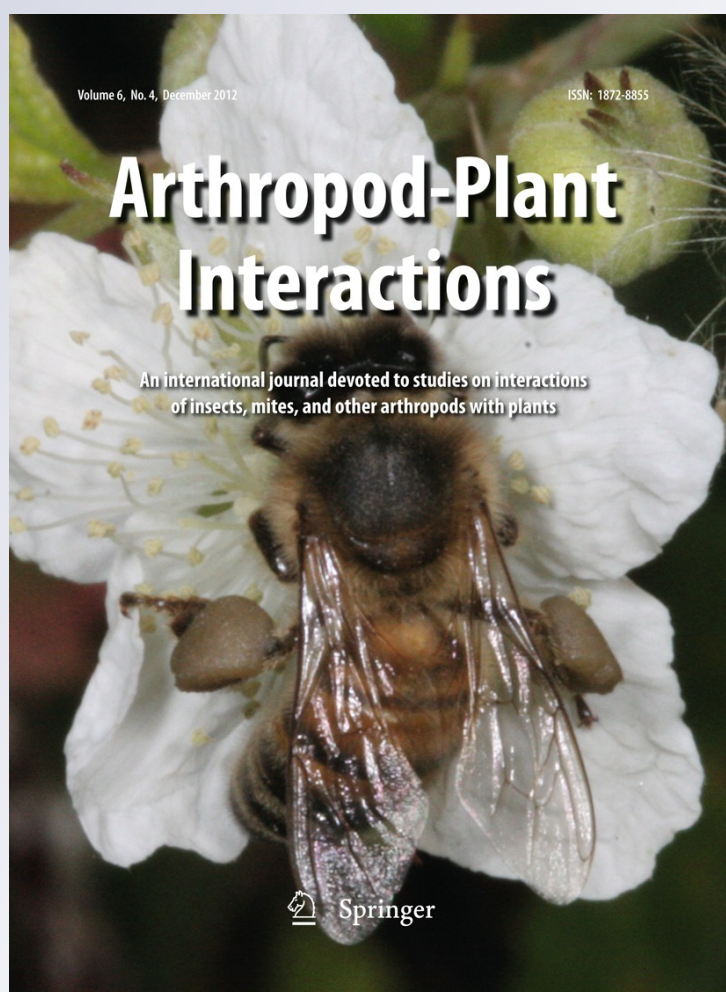
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Mutualism from the inside: coordinated development of plant and insect in an active pollinating fig wasp

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Abstract Recent studies on the obligate interaction between fig trees and their pollinating agaonid wasps have focused on population aspects and wasp–seed exploitation at the level of the inflorescence. Detailed studies on larval and gall development are required to more fully understand how resources are exploited and adaptations fine-tuned by each partner in nursery pollination mutualisms. We studied the larval development of the active pollinating fig wasp, *Pegoscapus* sp., and the galling process of individual flowers within the figs of its monoecious host, *Ficus citrifolia*, in Brazil. The pollinator development is strongly dependent on flower pollination. Figs entered by pollen-free wasps were in general more likely to abort. Retained, unpollinated figs had both higher larval mortality and a lower number of wasps. *Pegoscapus* sp. larvae are adapted to plant development, with two contrasting larval feeding strategies proceeding alongside gall development. The first two larval stages behave as ovary parasites. Later larval

stages feed on hypertrophied endosperm. This indicates that a successful galling process relies on endosperm, and also reveals why pollination would be a prerequisite for the production of high-quality galls for this *Pegoscapus* species.

Keywords Agaonidae · Coevolution · Larval biology · Mutualism stability

Introduction

Mutualisms are reciprocal, beneficial interactions between two species (Boucher et al. 1982). However, the processes by which one individual partner obtains benefits frequently involve costs to the other. This can lead to coevolution between partners for mechanisms that maximise benefits and minimise costs (Anstett et al. 1997). At the individual level, these coevolving processes can be antagonistic, resulting in a negative fitness correlation between partners. Paradoxically, such conflict at the individual level may result in a stable mutualism involving both partners (e.g. for the species or for the inter specific interaction itself, Margulis and Neelson 1989).

This conflict between mutualists is clear in the highly specialised plant–insect pollination mutualisms named nursery pollination mutualisms. In these mutualisms, plants reward pollinating insects with a proportion of their reproductive tissue in which to oviposit and to subsequently nourish their larvae. These oviposition sites vary between specific mutualisms and may involve flower receptacles or individual ovaries. In mutualisms in which pollinator larvae directly consume individual ovaries, the reward has a direct impact on plant reproduction because potential seeds are destroyed by the pollinator's offspring.

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At least seven nursery pollination mutualisms are known in which adult female insects are the exclusive pollen vectors of their host plant species (Dufay and Anstett 2003).

Although there have been numerous discussions on intrinsic conflicts within mutualisms (Bronstein et al. 2006; Dufay and Anstett 2003; Herre et al. 2008), little attention has been paid in recent decades to the basic biology of these systems. Studies have mainly focused on mechanisms that explain the stability of the mutualism, especially those based on host sanctions towards cheating partners (Herre et al. 2008; Jander and Herre 2010). Basic processes involved in nursery pollination are frequently inferred from indirect, incomplete information (e.g. Herre and West 1997).

In the intensively studied, obligate nursery pollination mutualism between fig trees (*Ficus*; Moraceae) and their pollinating agaonid wasps (Weiblen 2002), recent studies have focused on population aspects of pollinators and wasp–flower exploitation at the level of the inflorescence (Jander and Herre 2010; Jusselin and Kjellberg 2001; Tarachai et al. 2008). Studies of ovary–larva micro-structural interactions in fig flowers are still lacking. Existing data are scarce and are usually restricted to particular larval stages, failing to describe the whole developmental process (Condit 1932; Galil and Eisikowitch 1968; Grandi 1929; Johri and Konar 1956; Joseph 1958; Joseph 1984; Leclerc du Sablon 1907; Verkerke 1986, 1987).

Detailed studies on larval and gall development are required to fully understand how resources are exploited and to evidence fine-tuned adaptations of both partners in nursery pollination mutualisms (Anstett 2001; Jusselin and Kjellberg 2001; Pellmyr and Krenn 2002). The genus *Ficus* provides an ideal model system. Each of the approximately 750 extant *Ficus* species is pollinated by one or a few tiny species-specific agaonid wasp species (Weiblen 2002). Female pollinating wasps (foundresses) enter the 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 flower ovaries (Galil and Eisikowitch 1968) whilst simultaneously spreading the pollen they carry from their natal tree onto the stigmatic surface of the flowers (Jusselin et al. 2001, 2003). Pollination can be passive or active, depending on the species. In active pollination, the newly emerged female wasps collect pollen from the anthers in their natal fig and transfer it into specialized thoracic pollen pockets. Foundresses deposit pollen with their forelegs, giving them some control over pollen deposition within the fig. Conversely, in passively pollinated species pollen from the dehiscing anthers adheres to the wasp's exoskeleton (no wasp behaviour involved). In this case foundresses have little control over the pollen deposition process. In both cases, each larva develops within a single galled fig

ovule, meaning that each wasp offspring potentially translates into one lost seed.

There are selective benefits to the wasps in providing pollination services to actively pollinated fig species. First, in some cases, unpollinated figs are more likely to abort than pollinated ones (Tarachai et al. 2008; Jander and Herre 2010). Second, even though some pollinating species manage to survive and develop in unpollinated figs, brood size can be negatively affected (Jusselin and Kjellberg 2001; Jusselin et al. 2003; Tarachai et al. 2008). On the other hand, in passively pollinated species, wasp development seems to be independent from pollination. Experimental studies on passive pollinators have demonstrated that flowers containing eggs rarely receive pollen. This might be explained by flower morphological specialization—flowers more likely to produce seeds have elongated brush-shaped stigmas, while oviposited flowers have flat, short stigmas (Jusselin et al. 2003). Moreover, abortion rates of unpollinated figs did not differ from those of pollinated ones (Jander and Herre 2010). These differences between active and passive pollination suggest that plant–larva interactions may vary among fig species, with pollination modes subjected to different selective pressures. At the larval level, traits that allow the exploitation of fertilization-independent tissues should be favoured in those species in which eggs are just as likely to be laid in pollinated or unpollinated flowers (passive pollination and probably some active pollination systems), while traits that optimize the exploitation of fertilization-dependent tissues (i.e. the endosperm) would be favoured in cases in which flower fertilisation and egg laying coincide more frequently.

In order to better understand the mechanisms involved in plant–larva interactions we present data on the larval development of the active pollinating fig wasp, *Pegoscapus* sp., and the galling process of individual flowers within the figs of its monoecious host, *Ficus citrifolia*, in Brazil. We show that pollinator development is strongly dependent on flower pollination and that adaptations at the larval level are constrained by processes involved in seed development, such as pollination and fertilisation.

Materials and methods

Study species

Ficus citrifolia (subgenus *Urostigma*, section *Americana*) is a monoecious fig tree, and it is actively pollinated by an undescribed *Pegoscapus* species in Sao Paulo state (J. Y. Rasplus, Personal communications). Section *Americana* is sister group to section *Galoglychia* (Renoult et al. 2009). For this later section a detailed histological study already exists in *F. otoniifolia* (Verkerke 1986).

Wasps in pollinated flowers and seed development

We used *F. citrifolia* trees growing naturally on the campus grounds of Sao Paulo University, Brazil (21°10'S; 47°48'W). All work was performed between July 2007 and August 2008. We studied four cohorts of wasps, each from a different *F. citrifolia* tree.

From each tree, we isolated figs from 10 branches (approx. 150 figs) with white fabric bags before they were receptive to pollinators to prevent natural wasp infestation. When the figs became receptive, the bags were removed and a single foundress was introduced into each fig by carefully placing the wasp near the ostiole with a fine paintbrush and then waiting for it to enter. After all introductions were completed, the branches were re-bagged. The foundresses used for the experimental introductions were sourced from other nearby *F. citrifolia* trees that had crops of 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.

Our synchronized introductions allowed us to follow larval development by collecting the experimental figs at different times after introduction of the wasps. To do this, four to five figs per tree were collected every 2 days after 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 carefully cut open under 40× magnification stereomicroscope to sample 20 galled ovaries. Oviposited ovaries were identified by the scar made through the style by the female ovipositor. We collected the figs until the dissection process showed that the wasps had pupated. The dissected galls were photographed with a digital camera mounted on a Leica MZ16 stereomicroscope for description of larva/plant tissue development. Total body length along body midline and maximum width were measured for each larva 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 foundress introduction. Each group of ovules was processed according to standard dehydration and softening protocols and then embedded in Leica Histo-resin® (Gerrits 1991). Material was then sectioned manually with a Leica RM 2245

microtome into 6–8 µm sections. Serial sections were slide mounted and stained with toluidine blue 0.05 %, pH 4.4 (O'Brian et al. 1964). Each slide was then photographed using a digital camera attached to a Leica DM 4500 microscope. All histological slides and fig wasp samples were retained by R. A. S. Pereira. as voucher material.

Wasp performance in unpollinated flowers

To compare the performance of *Pegoscapus* sp. in pollinated and unpollinated flowers, we proceeded with controlled introduction of either pollen-loaded or pollen-free wasps in figs of three fig trees between April and November 2011. We introduced one single wasp in each of approximately 20 figs/treatment/tree. Control treatment consisted of figs in which no wasp was introduced. Experimental figs were isolated with white fabric bags before and after wasp introduction.

Pollen-loaded wasps were sourced from other nearby *F. citrifolia* trees where wasps were present. Pollen-free wasps were obtained by cutting open figs in which wasp were about to emerge. Galls containing fertilized female wasps that were about to emerge from their galls were removed using fine forceps and then isolated in tissue bags until wasp emergence.

Fig abortion was recorded for each treatment. Among the figs that did not abort we randomly sampled up to 10 figs to quantify the following variables: (1) number of galls, (2) number of bladders (empty galls that could result from larva death), (3) number of seeds, and (4) number of adult wasps.

Due to the high abortion rate of the figs entered by pollen-free wasps, it was not feasible to carry out histological study on unpollinated flowers.

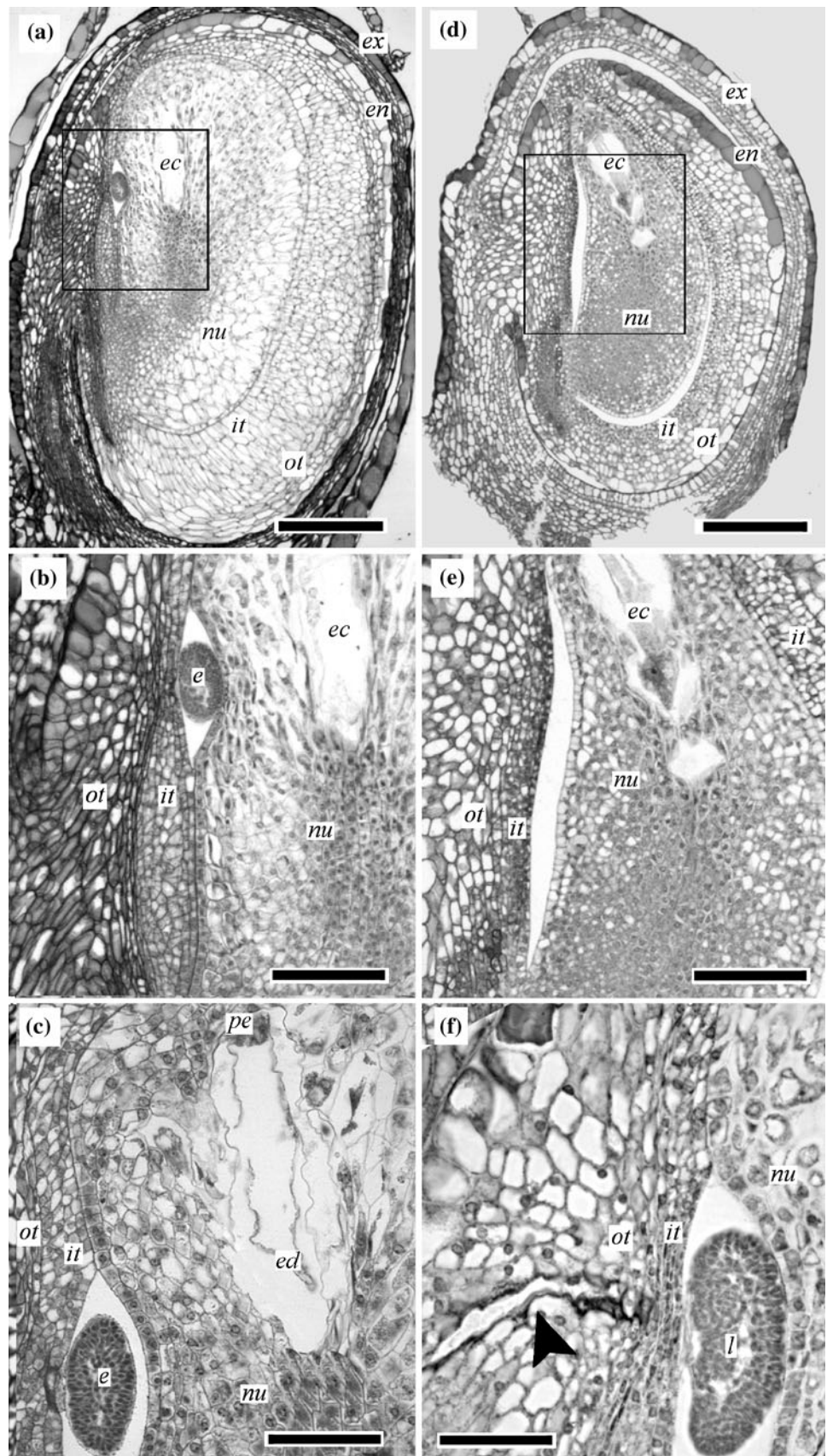
Results

Wasps in pollinated flowers and seed development

We observed four larval instars in *Pegoscapus* sp, as determined by changes in larval growth (Fig. 1 of electronic supplementary material). However, the detection of instar changes was not clear for all cohorts due to (1) the short duration of second and third instar, (2) the space constraints of the last two instars, limiting larval growth, and (3) the less-pronounced changes in size among instars in microhymenoptera (Damiens et al. 2001; Harvey et al. 1999, 2004).

Female pollinators always laid a single egg near the stylar canal entry, between the inner integument of the ovule and the nucellus (Fig. 1a, b). Part of the sections showed embryo sac cavities with a zygote at the micropylar

Fig. 1 Longitudinal sections of early stages of galled and normal seeds in *Ficus citrifolia*. **a** general view of gall flower; **b** detail showing the egg between nucellus and inner integument; **c** detail showing the egg and the plant proembryo; **d** general view of normal seed at the same age of (a–c); **e** detail of the framed area in (d); **f** detail of egg–first larval stage transition, scar left on by the wasp ovipositor is visible (arrow). *e* egg, *ec* embryonic cavity, *ed* endosperm, *en* endocarp, *ex* exocarp, *it* inner integument, *l* larva, *mes* mesoderm, *nu* nucellus, *ot* outer integument, *pe* seed proembryo, *st* flower style. Scale bars: **a, d**, 0.2 mm; **b, e**, 0.1 mm; **c, f**, 0.05 mm. (**a–d, f**) crop 1 (length = 38 days), 4 days after pollination; **e** crop 2 (length = 44 days), 4 days after pollination



region, indicating that the embryo sac was fertilised (Fig. 1c). When compared with unoviposited fertilised ovules, oviposited ovaries showed volume increase of nucellar and integumental cells, mainly those located distally from the wasp egg deposition site (Fig. 1d, e).

Larval feeding behaviour changed as larvae developed over time. These changes in feeding habit were accompanied by changes in their position inside galls according to plant embryogenesis. During late first and second larval stages, *Pegoscapus* sp. larvae fed on nucellus, whereas in subsequent stages, they fed mainly on galled endosperm.

Larvae hatched 2–6 days after oviposition. Larvae that had just hatched stayed in the position where the egg was laid (Fig. 1f). Older larvae in the first larval stage (approx. 12–14 days after oviposition) developed an oral cavity and a gut.

When the second larval stage was reached (approx. 16–18 days after oviposition), the larvae developed sclerotised mouthparts. At this time the larvae moved into the nucellus (Fig. 2a, b), Ovary expansion continued due to increasing volume of nucellar and integument cells, apparently without cell division (Fig. 2). In both galled and normal seeds we observed a globular proembryo (Fig. 2b–d). The endosperm of galled ovaries with second larval stages began to proliferate rapidly over the nucellus and showed a normal development that could be characterized as a nuclear-type development, with free nuclei forming a syncytium.

During the transition from the second to the third larval stage, the larvae migrated from a lateral nucellar position to a micropylar one, where the embryo sac cavity was initially placed (Fig. 3a). The plant embryo was probably eliminated at this transitory time as no plant embryos were found in conjunction with third stage larvae in either dissected material or histological sections.

During the third larval stage (beginning approx. 20–24 days after oviposition), the endosperm of galls began an abnormal cellularisation, producing large vacuolated cells, some with more than two nuclei (Fig. 3b). Third stage larvae began to feed on this tissue. At this stage the gall differed conspicuously from normally developing seeds. Coating wall tissues of the gall looked thinner, with fewer cell layers than a normal seed (Fig. 3a, c). Normal seeds at the same developmental stage presented an endosperm still in the process of cellularisation, with no signs of abnormal cell growth (Fig. 3c, d).

Fourth stage larvae (beginning approx. 26–28 days after oviposition) were larger and continued feeding on abnormal endosperm until occupying the whole ovarian cavity. Larvae then changed to pre-pupae and finally to pupae, when metamorphosis took place prior to eclosion (approx. 38–42 days after oviposition).

Wasp performance in unpollinated flowers

Despite substantial variation among crops in abortion rates, wasp development was always negatively affected by lack of pollination (Table 1). In two trees in the pollen-free treatment (Table 1a), the abortion rate of unpollinated figs was 84–100 % compared with 43–55 % for the pollinated ones (Table 1b). For the third tree, no abortion occurred irrespective of the treatment (Table 1a, b). However, in figs that did not abort, larval mortality (number of bladders) was about 4–10 times higher and adult wasps emerged from unpollinated figs were half of those from pollinated figs. Wasp sex ratio was not affected. All figs under the control treatment aborted.

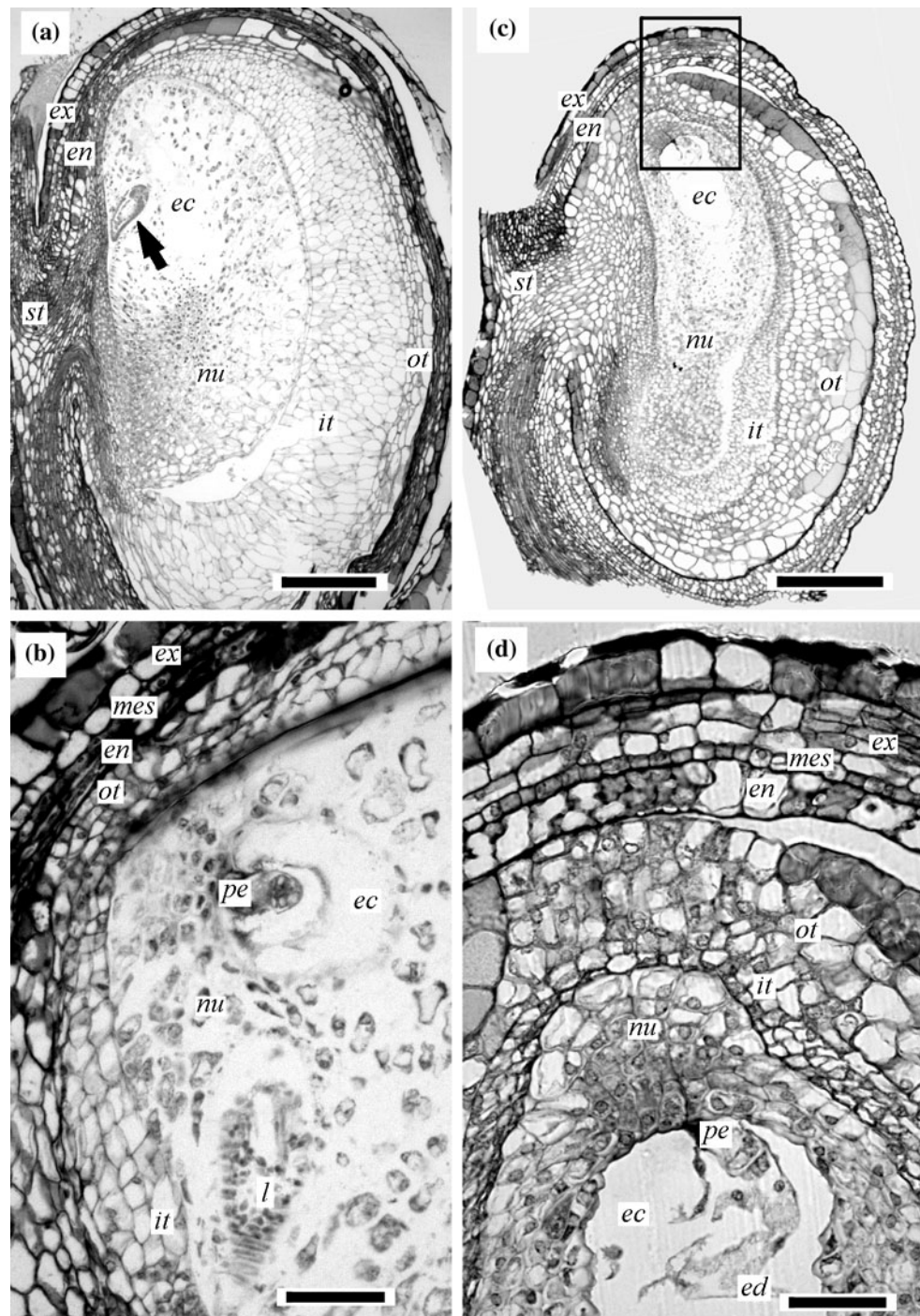
Discussion

We demonstrate here experimentally that the development of *Pegoscapus* sp. is facilitated by ovule fertilization. Figs entered by pollen-free wasps were in general more likely to abort. Retained, unpollinated figs produced fewer wasps but presented many bladders. We observed that the size of flower ovaries increased rapidly after oviposition, reaching their final volume in few days. This ovary growth was due to the increase of volume of nucellar and integument cells, apparently without cell division. We suggest that this growth could be a tissue reaction to substances injected by the female wasp during the oviposition.

Our histological results suggest that *Pegoscapus* sp. has specialized on the induction and consumption of hypertrophied endosperm. During the two first instars, the larva feeds on the nucellus (i.e. on a tissue that does not result from the double fertilization). Nevertheless, later two larval instars depend on tissues resulting from the double fertilization. This phase of wasps development is probably the phase during which selection for active pollination is enacted. In the absence of pollination, wasp mortality should occur at the third larval instar, when tissues from the double fertilization begin to be exploited and ovule has reached its final dimension. This is coherent with the observation of numerous enlarged but empty ovaries called bladders in the fig literature.

The galling process observed for *Pegoscapus* sp. in *F. citrifolia* was similar to that described for *Courtella gabonensis* in *F. ottoniifolia* (section *Galoglychia*, sister group of section *Americana* to which *F. citrifolia* belongs) and *Kradibia gestroi* in *F. asperifolia* (section *Sycidium*) (Verkerke 1986, 1987). Early gall induction in all three species seems to involve rapid increase in volume of nucellar and integument cells during the first days following egg deposition (Verkerke 1986, 1987). In *F. citrifolia* and *F. ottoniifolia* this phenomenon seems to affect

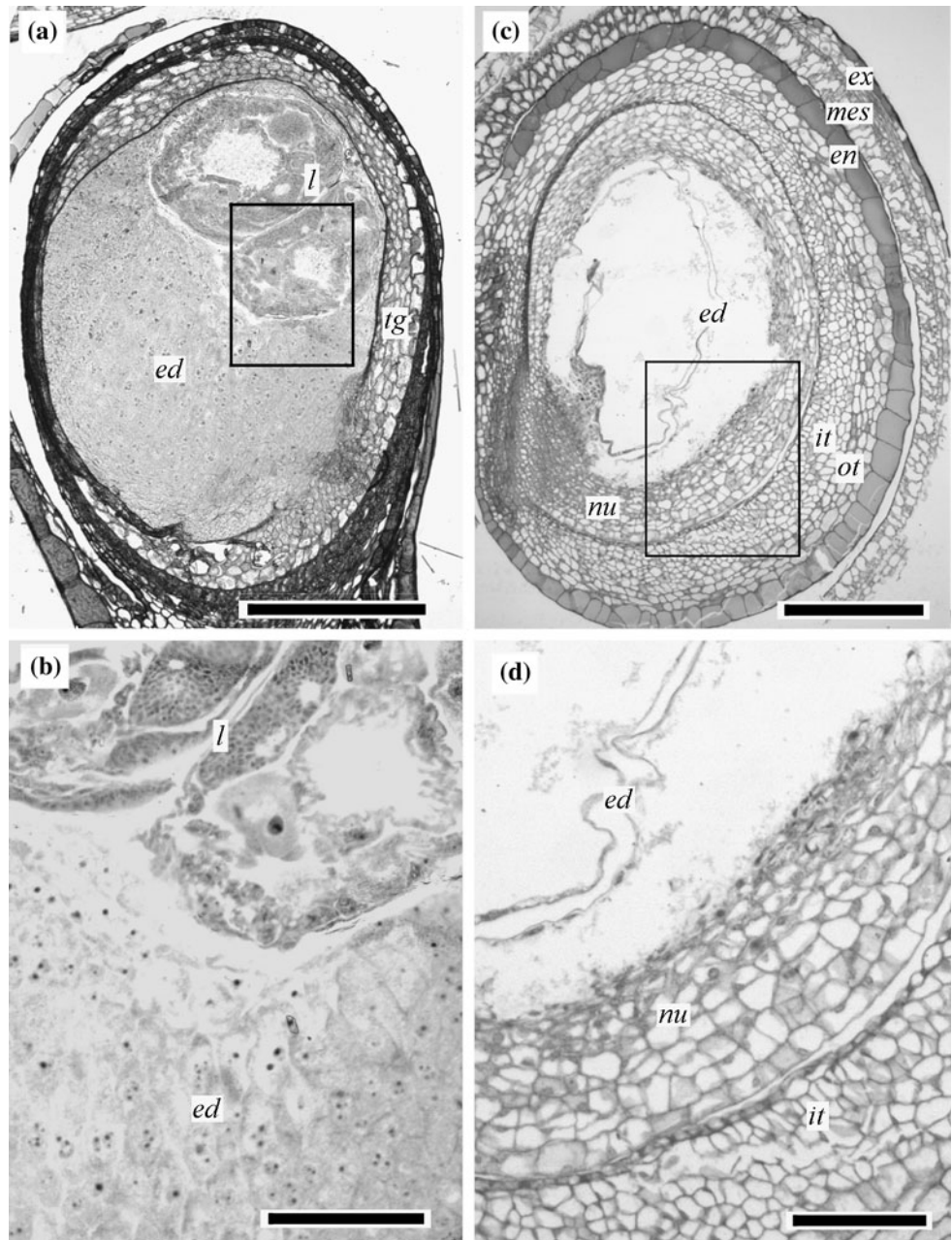
Fig. 2 Longitudinal sections of galled and normal seeds of *Ficus citrifolia*. **a** general view of gall flower with second larval stage (*arrow*); **b** detail showing second larval stage and plant proembryo; **c** general view of normal seed at the same age of (**a**); **d** detail of framed area in (**c**). *e* egg, *ec* embryonic cavity, *ed* endosperm, *en* endocarp, *ex* exocarp, *it* ovule inner integument, *l* larva, *mes* mesoderm, *nu* nucellus, *ot* ovule outer integument, *pe* globular seed proembryo, *st* flower style. Scale bars: **a, c**, 0.2 mm; **b, d**, 0.05 mm. **a, c** crop 2 (length = 44 days), 16 days after pollination; (**b–d**) crop 1 (length = 38 days), 16 days after pollination



cells located distally from the egg. Comparing our results to Verkerke's (1986) diagrammatic representation suggests the endosperm position in his figure 7b is misinterpreted. No data are available about the importance of pollination for successful gall induction for these species. Interestingly in both *Ficus citrifolia* and in many species of section *Galoglychia* some non-pollinating fig wasp species (NPFW) are able to gall receptive unpollinated fig flowers. The neotropical NPFW *Idarnes* group *flavicollis* colonise

figs about the same time pollinators do and are able to successfully gall unpollinated ovaries and prevent the abortion of unpollinated figs (Elias et al. 2008, 2012). *Idarnes* group *flavicollis* wasps seem to be efficient gall makers, inducing abnormal proliferation of nucellus cells a few days after egg deposition (S. Jansen & R. A. S. Pereira, unpublished data). Similarly many fig species of section *Galoglychia* are colonised by *Philocaenus*, a genus of wasps that enters receptive fig to oviposit and successfully

Fig. 3 Longitudinal sections of advanced stages of galled and normal seeds of *Ficus citrifolia*. **a** gall with third larval stage and hypertrophied endosperm; **b** detail showing the larva and hypertrophied endosperm; **c** seed at the same age of **(a)**; **d** detail of the framed area in **(c)**. ed endosperm, en endocarp, ex exocarp, it ovule inner integument, *l* larva, *mes* mesoderm, *nu* nucellus, *ot* ovule outer integument. *Scale bars:* **(a)**, **(c)**, 0.5 mm; **(b)**, **(d)**, 0.05 mm. **(a–d)** crop 1 (length = 38 days); **(a–b)** 28 days after pollination; **(c–d)** 24 days after pollination



induces fig development (Compton 1993). Comparing how these wasps manage to successfully gall receptive female flower may help understand the evolutionary constraints associated with oviposition in receptive female fig flowers.

The first step of larva–plant interaction (i.e. egg deposition exactly between internal integument and nucellus) seems to be conserved in *Ficus* (Cunningham 1889; Leclerc du Sablon 1907; Condit 1932; Johri and Konar 1956; Verkerke 1986, 1989; Ghana and Compton in press; Jansen-Gonzalez and Sarmiento 2008). However, the later steps of the gall process seem to respond to different selective pressures. Data on neotropical fig species (Jander and Herre 2010) show that abortion rates of unpollinated

figs are no greater in passively pollinated fig species (i.e. section *Pharmacosycea*) and that abortion ratio may vary widely among actively pollinated species (i.e. section *Americana*).

The physiological mechanism by which pollinating fig wasps may induce retention of unpollinated figs is unknown. It has been suggested that fig trees may abort unpollinated figs as a sanction against cheaters, i.e. against wasps that fail to perform the mutualistic pollination service (Jander and Herre 2010). Our results may allow a more mechanistic analysis of the process. Indeed, in *F. citrifolia*, in most flowers, according to our histological observations, there is a phase when the wasp larvae shift

Table 1 Abortion rate and variables quantified on the experiment of *Pegoscapus* sp. performance in pollinated and unpollinated figs of *F. citrifolia*

Fig tree	<i>N</i>	Abortion (%)	<i>n</i>	Seeds	Bladders	Wasps	Sex ratio ^a
(a) Pollinated figs							
1	23	43	10	131.3 ± 31.3	15.9 ± 8.5	197.1 ± 50.8	0.10 ± 0.02
2	17	53	8	136.5 ± 22.4	52.6 ± 18.1	179.6 ± 22.8	0.06 ± 0.02
3	21	0	10	178.8 ± 33.1	13.7 ± 6.2	142.3 ± 31.3	0.08 ± 0.04
(b) Unpollinated figs							
1	19	100	0	–	–	–	–
2	19	84	3	0.0	199.0 ± 4.5	72.33 ± 16.8	0.07 ± 0.04
3	20	0	9	0.0	107.6 ± 49.7	97.8 ± 40.4	0.08 ± 0.05*
(c) Control (no wasp)							
1	20	100	0	–	–	–	–
2	20	100	0	–	–	–	–
3	20	100	0	–	–	–	–

Values are mean ± standard deviation. *N* = number of manipulated figs; *n* = number of figs where variables were quantified

* We excluded one outlier observation (Sex ratio = 0.98, *n* = 114 wasps)

^a Total male wasps : total wasps

from feeding on host plant tissue to feeding on the endosperm. In unpollinated figs wasp larvae are forced to feed on alternative resources. As *Pegoscapus* sp. has specialized on galling and consuming endosperm, unpollinated figs lead to high larval mortality (i.e. increase number of bladders). Lack of developing seeds and reduced numbers of developing larvae may lead to frequent fig abortion because of a reduced sink effect (not enough developing ovules). In a passively pollinated species, *F. maxima*, it was shown that most larvae develop in unfertilised ovules because features of the stigma lead to pollination and fertilisation of longer styled flowers that produce seeds and not of shorter style flowers that host wasp larvae (Jousselin et al. 2004). Hence in that passively pollinated fig, and probably in most or all passively pollinated species, wasps are selected for their capacity to gall and develop in unfertilised ovaries. Lack of pollination of the fig should not affect survival probability of the larvae and therefore unpollinated, oviposited figs should not abort. Actively pollinating fig wasp species may abundantly pollinate the figs so that most larvae will develop in fertilised ovules. In that case we may expect the wasps to survive poorly in unfertilised ovules because of lack of selection for this capacity. Reciprocally some actively pollinating species pollinate less systematically the fig leading to frequent development of wasp larvae in unfertilised ovules (Jousselin et al. 2003). Such wasps are selected to retain the capacity to develop in unfertilised ovules. Hence in fig-fig wasp systems involving active pollination, intensity of pollination, reduced capacity to successfully gall non fertilised ovules and abortion rates of unpollinated figs are predicted to be positively correlated traits due to selection

on the wasp. There is no theoretical requirement for this correlation to be due to sanctions imposed by the tree on non-pollinating wasps.

Moreover, the hypothesis of fig abortion being a selected mechanism against cheaters seems to be of little empirical support. As pollinating fig wasps have a shorter generation time, it is very probable they should be selected to overcome plant defences and become more efficient at inhibiting fig abortion. Documenting the larva-plant interaction in detail allowed us to better understand the selective pressures involved on the fig-fig wasp mutualism. Widening this approach to fig species where pollinators have higher fitness in unpollinated flowers will improve the comprehension of partner traits that lead to mutualism stability.

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ELECTRONIC SUPPLEMENTARY MATERIAL

Mutualism from the inside: coordinated development of plant and insect in an active pollinating fig wasp

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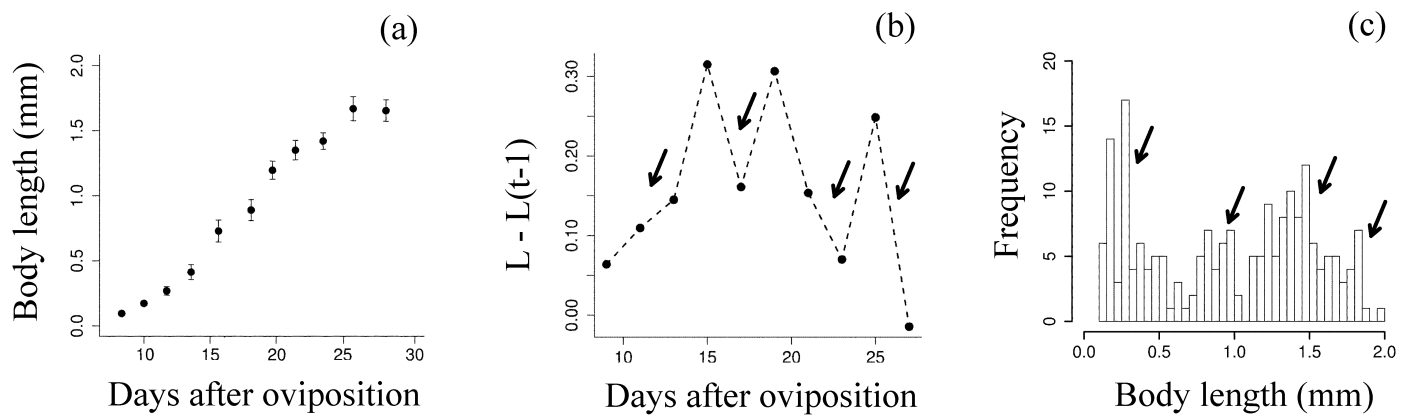


Fig. 1 Larval stage determination by growth curves, growth acceleration and frequency plots for *Pegoscapus* sp., in *Ficus citrifolia*. Data correspond to one out of the four introduction experiments. (a) Means \pm SE of larva length (N=20 larvae per day); (b) Acceleration of larval growth. Valleys correspond to each of the four larval stages. Acceleration was estimated from the average larva size of two subsequent samples [$size_t - size_{t-1}$, where $size$ is the average body length (n=20) and t is time]; (c) Frequency distribution of body size. Arrows correspond to a larval stages.