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Extrafloral nectaries as a deterrent mechanism against seed predators in the chemically protected weed *Crotalaria pallida* (Leguminosae)

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Abstract In several plants, extrafloral nectaries (EFN) are located close to the reproductive structures, suggesting that ants may act as a defence against specialized seed predators that overcome chemical defences. Alternatively, ants may also deter herbivores in a generalized manner, thereby protecting the whole plant. In this work, we examined the relationship between the chemically protected weed *Crotalaria pallida* Ait. (Leguminosae) that bears EFN, its specialized seed predator, the larvae of the arctiid moth *Utetheisa ornatrix* L. (Arctiidae) and ants. We tested two hypotheses related to the type of deterrence caused by ants. The Seed Predator Deterrence Hypothesis predicts that ant deterrence is directed primarily towards herbivores that destroy seeds and other reproductive structures, without attacking herbivores on vegetative structures. The General Deterrence Hypothesis states that ants are general in their effects, equally deterring herbivores in vegetative and reproductive structures. Our results supported the predictions of the Seed Predator Deterrence Hypothesis, namely, that (i) ant activity on EFN was related to the vulnerability of reproductive structures to attack by *U. ornatrix*; (ii) ant patrolling was restricted almost entirely to racemes; (iii) ants removed termites used as baits more frequently on racemes than on leaves; and (iv) *U. ornatrix* larvae were often expulsed from the racemes. These results indicate that EFN can act as another deterrent mechanism in chemically protected plants by promoting the expulsion of specialist seed predators.

Key words: Arctiidae, *Camponotus*, chemical defence, deterrence, *Ectatomma*, Formicinae, Ponerinae, pyrrolizidine alkaloids, seed predation, *Utetheisa ornatrix*.

INTRODUCTION

Ant-guard systems are mutualistic interactions between ants and plants in which the ants provide defence against the plants' natural enemies and the plants provide rewards, such as extrafloral nectar (Bentley 1977; Elias 1983; Beattie 1985; Beattie & Hughes 2002). Extrafloral nectar is produced by secretory structures located on leaves, twigs or the external surface of flowers. These structures, which are referred to collectively as extrafloral nectaries (EFN), are not directly related to pollination, and ants are their main visitors (Elias 1983; Beattie 1985). The EFN-related activity of ants may benefit plants by (i) reducing leaf herbivory (Koptur 1979); (ii) reducing twig destruction (Oliveira 1997); (iii) enhancing fruit and seed production (Oliveira *et al.* 1999; Sobrinho *et al.* 2002); (iv) reducing the visitation of ants to floral nectaries, thereby enhancing the success of pollination (Wagner & Kay 2002); and (v) reducing seed predation and flower consumption (Pickett & Clark 1979; Keeler 1980, 1981, 1985; Del Claro *et al.* 1996; Oliveira 1997).

In many plants, EFN are located close to reproductive structures, suggesting that ant deterrence is directed primarily towards herbivores that destroy seeds and other reproductive structures. This pattern is observed in several plant species of the genus *Crotalaria*. In *C. pallida*, ants are likely to be important defenders of the reproductive structures when these are attacked by the specialized predispersal seed predator *Utetheisa ornatrix* that is able to overcome the plant's chemical defences (see Eisner & Meinwald 1995 for a review). However, reproductive structures are located at the end of large branches with leaves. Hence, ants may also visit the vegetative parts of plants, thereby deterring herbivores in a generalized manner. Based on these two putative defensive roles for ants in the *C. pallida-U. ornatrix* system, we proposed two hypotheses to explain this behaviour,

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namely, the Seed Predator Deterrence Hypothesis and the General Deterrence Hypothesis.

The Seed Predator Deterrence Hypothesis (SPDH) postulates that ant deterrence is directed primarily towards herbivores that destroy seeds and other reproductive structures, without attacking herbivores on vegetative structures. The predictions of the Seed Predator Deterrence Hypothesis investigated in this study were that (i) ant activity on EFN was related to the vulnerability of the phenological phase of the raceme to seed predators; (ii) ant patrolling was restricted to racemes; (iii) ants removed termites used as baits only from racemes and not from leaves; and (iv) *U. ornatrix* larvae were often expulsed from the racemes. Alternatively, the General Deterrence Hypothesis (GDH) states that ants are general in their effects, equally deterring herbivores on vegetative and reproductive structures. The predictions of the General Deterrence Hypothesis investigated here were that (i) ant activity on extrafloral nectaries was unrelated to the phenological phase of the raceme; (ii) ant patrolling occurred over all plant parts; and (iii) ants removed termites used as baits equally from racemes and leaves. Assessment of the fourth prediction of the SPDH was unnecessary in testing the GDH since it concerned seed specialist predators.

METHODS

Study site and species studied

Fieldwork was done from January to May 2002 in Campinas (22°54′20″S, 47°03′39″W), São Paulo State, south-eastern Brazil. All observations and experiments were done on sunny days, between 08.00 h and 11.30 h. The study site was an area of grassland with several weed species, including three *Crotalaria* species: *C. lanceolata* Mey, *C. incana* L and *C. pallida*. The latter was the most common species of the genus at the study site and occurred in dense aggregations (Guimarães 2003).

Crotalaria pallida Ait. (Leguminosae), a pantropical EFN-bearing weed that is widespread in south-eastern Brazil (Lorenzi 2000), varies in size from a small herb <0.5 m in height to a large bush 2–3 m tall (Polhill 1982). Each of the main branches has several leaves and terminates in a raceme. *Crotalaria pallida* produces from one to dozens of racemes, each with 30–50 flower buds, flowers or pods, and the same individual may simultaneously have racemes with flower buds, flowers or ripe pods. The EFN of *C. pallida* are located in the racemes, at the base of each flower or pod. *Crotalaria pallida* bears racemes throughout the year, although the peak of fruit production is in the wet season (Ferro 2001). Aerial plant parts contain different amounts of pyrrolizidine alkaloids (Ferro 2001), compounds probably related to defence against herbivores (van Dam *et al.* 1995). Indeed, a recent study suggested that postdispersal seed predators of *C. pallida* such as harvester ants are deterred by the presence of these alkaloids in ripe seeds (Guimarães 2003).

The main seed predators of *C. pallida* at the study site were larvae of the arctiid moth *Utetheisa ornatrix* L., which accounted for over 90% of the records of infested pods (Ferro 2001), and infested 16.9% of unripe pods ($n = 9345$ pods, Guimarães 2003). *Utetheisa ornatrix* larvae attack many *Crotalaria* species by consuming unripe seeds (Eisner & Meinwald 1995), although occasionally flowers, flower buds, and leaves are also consumed (Ferro 2001). This moth species is adapted to sequester the pyrrolizidine alkaloids found at a higher concentration in unripe seeds than in other parts of *Crotalaria*, and uses them for defence against predators and for the biosynthesis of a male sexual pheromone (Eisner & Meinwald 1995). During five years of study, other herbivores were rarely observed feeding on *C. pallida* leaves at the study site.

Ant activity on extrafloral nectaries and the phenological phase of the raceme

Field observations were used to determine whether there was a relationship between ant activity on EFN and the phenological phase of the raceme. The SPDH predicts that ant activity will be higher when the reproductive structures are more vulnerable to attack by seed predators. *Utetheisa ornatrix* does not attack ripe pods, but occasionally forages on leaves, flowers and, more often, on unripe pods of *C. pallida* (Ferro 2001). Hence, if the SPDH was correct, we would expect ant activity to be higher when the racemes bore flowers and/or unripe pods. However, if the GDH was correct, we would expect ant activity to be unrelated to the phenological phase.

Thirty racemes in each phenological stage (flower buds, flowers, unripe pods and ripe pods) were sampled, each one from a different individual of *C. pallida*. More than one phenological stage is commonly observed in a single raceme of *C. pallida*. To control for this variation, only racemes in which all reproductive structures (e.g. flowers) were at the same phenological stage were sampled. The presence of ants and the proportion of racemes in which there was at least a single ant were recorded. Bootstrapping procedures (10 000 randomizations) were used to generate a 95% confidence interval for the observed proportion of racemes in which there were ants. The confidence limits were determined using the percentile method (Manly 1997).

Ant patrolling and plant parts

Field observations were made to determine whether there was a relationship between ant patrolling and plant parts. The SPDH predicts that ant patrolling will be restricted to racemes (EFN-bearing structures) and that leaves (structures without EFN) will not be patrolled, whereas the GDH predicts that ant patrolling will be equal among plant parts. One leaf and one raceme with unripe pods were randomly assigned in each plant ($n = 30$), and both structures were observed simultaneously for 15 s. The leaves and racemes are similar in area. All ant species that visited the racemes and leaves, and their abundance, were recorded, after which the worker ants were collected and preserved in 70% ethanol for later identification. Voucher specimens were deposited in the Museu de Zoologia da Universidade de São Paulo (MZUSP). We recorded the frequency of plant parts patrolled by ants and used the binomial test to test the differences in the patrolling of ants between the racemes and leaves.

Removal of termites used as baits by ants and plant parts

An experiment was performed to determine whether there was a relationship between the removal of termites used as baits and plant parts. Termites are often used to investigate the deterrence of herbivores by ants (Freitas & Oliveira 1996). The SPDH predicts higher removal of termites from racemes (EFN-bearing structures) than from leaves (structures without EFN), whereas the GDH predicts that termite removal will be equal among plant parts. Live termite workers (*Neocapritermes opacus* Hagen; Termitidae) were glued by the dorsum to selected sites using white glue (Tenaz$^{\text{TM}}$), as described by Freitas and Oliveira (1996). Eighty plants were randomly assigned to four experimental groups ($n = 20$ plants per group): (1) one termite worker was glued in the middle of the highest unripe pod; (2) one termite worker was glued on a leaf, ~10 cm from the nearest EFN; (3) as in group 1, except that the ants were excluded by protecting the base of the raceme with atoxic wax (Tanglefoot$^{\text{TM}}$); and (4) as in group 2, except that the ants were excluded by protecting the petiole of the leaf with atoxic wax. In the latter two experimental groups, all of the leaves and twigs that connected the raceme or leaf to other plants were removed, thereby eliminating any bridges for the ants. Experimental groups (3) and (4) served as controls for the effects of other predators that were not excluded by the experimental design, including jumping spiders and wasps. All of the plants were checked after 2 h and the number of termites removed was recorded and compared among the experimental groups using Fisher's exact test (Zar 1999). No termite was attacked in the two control groups, and the final analyses compared only groups 1 and 2. Whenever possible, the behaviour of ants attacking the termites was recorded.

Expulsion of the seed predator from the racemes

The last experiment was designed to investigate a particular prediction of the SPDH, namely, that ants efficiently deter the specialist seed predator, in this case, larvae of the moth *U. ornatrix*. Ants apparently cannot attack larvae that are inside the pod (Ferro 2001). However, larvae of all instars were frequently seen outside the pods, moving from one pod to another, and sometimes from one raceme to another. In this experiment, we investigated whether two ant species, namely, *Camponotus blandus* Smith (Formicinae) and *Ectatomma quadridens* Fabr. (Ponerinae), efficiently protected the pods against the last instar larvae of *U. ornatrix*. We choose these two ant species because (i) both species are easily recognized in the field and also easily manipulated in experiments; and (ii) *Camponotus* (Formicinae) and *Ectatomma* (Ponerinae) are very different in their attack behaviour (see Results) and morphology, allowing us to investigate if *U. ornatrix* is deterred by very different ant species. Only one raceme was used per plant and only one *U. ornatrix* was placed on each raceme (11 racemes with *C. blandus*, and 10 with *E. quadridens*). The racemes were monitored continuously until (i) an ant preyed upon the larva; (ii) the larva was expulsed from the raceme by ants; or (iii) the larva entered an unripe pod.

RESULTS

The activity of ants in the EFN was highest in racemes during flowering and in the unripe pod stage, decreasing in racemes with ripe pods (Fig. 1). Ant activity

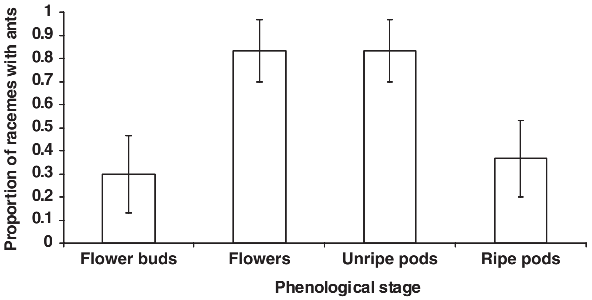

Fig. 1. Number of racemes of *Crotalaria pallida* with ants according to the phenological stage ($n = 30$ racemes in each phenological stage). The bars indicate the 95% confidence interval estimated using bootstrapping procedures.

Table 1. The frequency of ants on individuals of *Crotalaria pallida* ($n = 30$) in south-eastern Brazil

Ant species	Frequency
FORMICINAE	
Brachymyrmex sp.	3
Camponotus blandus Smith	7
Camponotus crassus Mayr	10
Camponotus leydigi Forel	2
Camponotus personatus Emery	1
Paratrechina sp.	4
Unindentified species	2
MYRMICINAE	
Crematogaster sp.	1
PONERINAE	
Ectatomma quadridens Fabr.	3
PSEUDOMYRMECINAE	
Pseudomyrmex sp.	[†]

[†] Observed on racemes, but not during the sampling period.

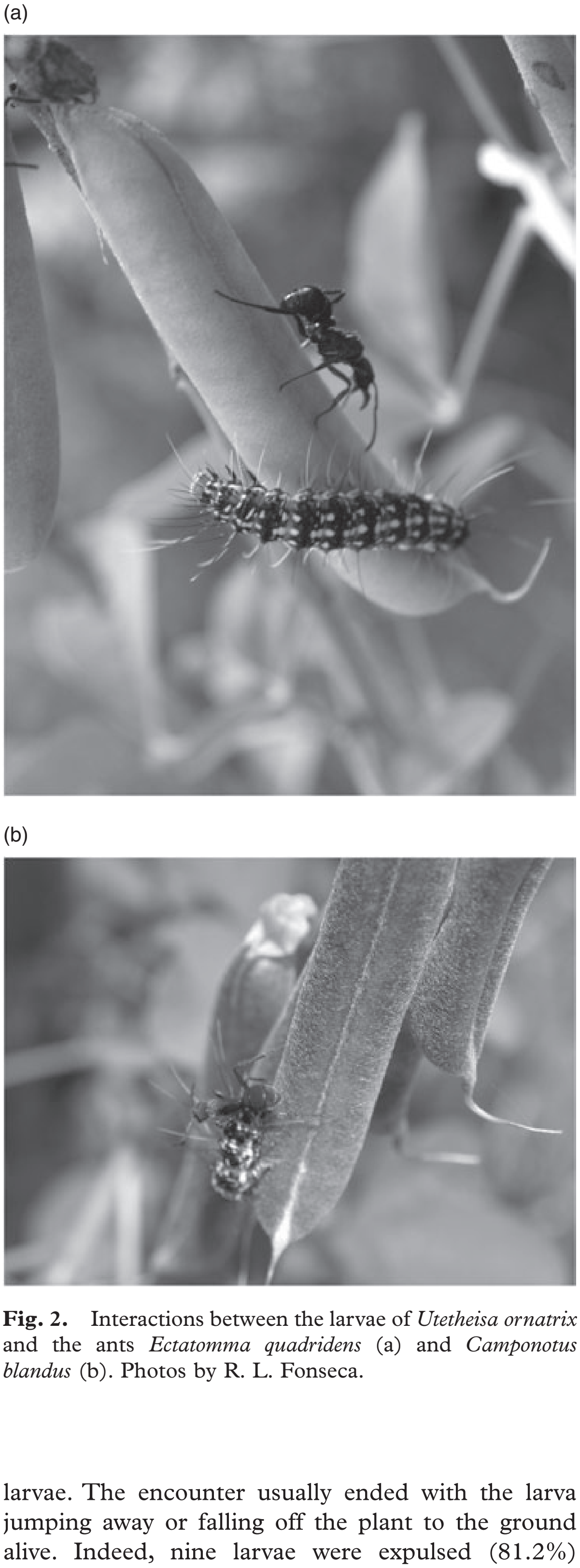

Fig. 2. Interactions between the larvae of *Utetheisa ornatrix* and the ants *Ectatomma quadridens* (a) and *Camponotus blandus* (b). Photos by R. L. Fonseca.

varied significantly between leaves and pods: ants occurred on 28 racemes with unripe pods (94%, 4.6 ± 1.4 ants per raceme, mean ± SE) and only one leaf (3%) had a single ant (binomial test, $P < 0.0001$). Eight ant species from three subfamilies were recorded visiting racemes of *C. pallida* (Table 1). The most frequent ant species were *Camponotus crassus* and *Camponotus blandus*. In addition to the species recorded during sampling, individuals of *Pseudomyrmex* sp. (Pseudomyrmecinae) were occasionally observed on racemes in the field. Twenty-three racemes showed only one ant species (82.1% of the racemes with ants), and five racemes (17.8%) had two species in their EFN at the same time.

The termites used as baits glued to pods were removed by ants significantly more frequently (85%, $n = 20$) than those glued to leaves (26%, $n = 19$) (Fisher's exact test, $P < 0.05$). Ant species varied in their behaviour when attacking the termites. Small formicine ants, such as *Brachymyrmex* and *Paratrechina,* recruited a large number of workers (>10 individuals) and slowly removed small pieces of the termite, whereas large ants, such as *Camponotus*. and *E. quadridens*, rarely recruited other ants and often removed the entire termite. *Ectatomma quadridens* always used its mandibles to attack the termites whereas *Camponotus* used both its mandibles and acid secretions from the acidopore.

Finally, *C. blandus* and *E. quadridens* attacked *U. ornatrix* last-instar larvae and deterred them in a similar way. The larvae usually ran away from the ants by moving quickly from one pod to another (Fig. 2). However, in most cases, some ants (usually one or two in both species) repeatedly attacked the larvae. The encounter usually ended with the larva jumping away or falling off the plant to the ground alive. Indeed, nine larvae were expulsed (81.2%) from the raceme by *C. blandus* ($n = 11$), while seven larvae were expulsed (70.0%) from the raceme and one larva was preyed upon (10.0%) by *E. quadridens* ($n = 10$).

DISCUSSION

Our results completely supported three of the four predictions of the Seed Predation Deterrence Hypothesis, namely, that (i) ant activity on EFN was related to the vulnerability of the phenological phase of the raceme to predation by *U. ornatrix*; (ii) ant patrolling was almost totally restricted to racemes; and (iii) *U. ornatrix* larvae were often expulsed from the racemes. Our second prediction that ants removed termites used as baits solely from reproductive structures was not completely supported since ants also removed termites from leaves. However, the removed rate was significantly higher from the reproductive structures than from the leaves.

Although the protection of foliar structures may enhance plant fitness (Koptur 1979; Smiley 1985), seeds are the ultimate product of plant fitness and influence the plant's reproductive success more directly than leaves (Janzen 1969, 1971). Hence, we interpret our results of the three-fold greater removal of termites used as baits from racemes as partial evidence that the deterrence of seed predators is the main function of extrafloral nectaries in *C. pallida*. Our data consistently indicate that protection of the reproductive structures in *C. pallida* is the main function of extrafloral nectaries in this plant. Based on these findings, we propose a scenario for the evolution of this pattern in ant–plant–herbivore interactions that involves three processes: (i) the ability of seed predators to overcome chemical defences; (ii) the association of ant-guarding with extrafloral nectaries to deter seed predators; and (iii) the association of extrafloral nectary activity and position.

As with other species of the genus (Polhill 1982) the leaves and particularly the seeds of *C. pallida* contain pyrrolizidine alkaloids (Sharma *et al.* 1965; Johnson *et al.* 1985; Ferro 2001) that may prevent seed predation and herbivory (Bentley *et al.* 1984; Dreyer *et al.* 1985; van Dam *et al.* 1995; de Boer 1999; Hägele & Rowell-Rahier 2000; Guimarães 2003). However, in some populations of *C. pallida*, predispersal seed predation is positively correlated with the concentration of alkaloids in the seeds, suggesting that predispersal seed predators of *C. pallida* can overcome the plant's chemical defences and use these alkaloids for their own protection (Moore 1978). Indeed, *U. ornatrix* is a classic example of a predispersal seed predator that overcomes plant chemical defences. In this case, the larvae sequester pyrrolizidine alkaloids and use them for their own protection and other biological functions (see Eisner & Meinwald 1995 for a review). In this ecological scenario, secondary compounds do not deter specialized herbivores such as *U. ornatrix*, but instead, serve as a source of important compounds that enhance their fitness (Eisner & Meinwald 1995). As a result, alternative deterrent mechanisms, such as ant-based defences, may have evolved in these plants to deter specialized seed predators as an alternative to chemical defences. Although no study has investigated whether this shift occurred or is occurring in *C. pallida*, Ferro (2001) found a low concentration of pyrrolizidine alkaloids in *C. pallida* seeds compared with other congeneric species. In other ant-guard systems, such as the interactions between myrmecophytes and ants, a reduction in chemical defences associated with an increase in extrinsic defences by ants has also been reported (e.g. Janzen 1966; Fonseca 1994; Heil *et al.* 2000; but see Heil *et al.* 2002).

Our results showed that most *U. ornatrix* larvae were expulsed from racemes by ants of the two species investigated, namely, *C. blandus* and *E. quadridens*. However, although ants expulsed *U. ornatrix* from the plant, predation events were rare. Herbivores specialized in consuming parts of EFN-bearing plants have defensive strategies against deterrence by ants (Heads & Lawton 1985; Freitas & Oliveira 1992, 1996; Eubanks *et al.* 1997; Di Giusto *et al.* 2001; Machado & Freitas 2001). These defences include frass chains (Freitas & Oliveira 1996; Machado & Freitas 2001), distasteful compounds (Heads & Lawton 1985; Di Giusto *et al.* 2001), the construction of shelters (Eubanks *et al.* 1997), and hiding inside tied leaves (Heads & Lawton 1985). All of these defences allow herbivores to survive and continue consuming the same host plant. In contrast, *U. ornatrix* uses another defence against predation (Heads & Lawton 1985): it jumps off the plant when attacked by *Camponotus* and *Ectatomma* ants. The act of jumping off a plant implies that the larva will not feed on the seeds of the same plant again, especially because *C. pallida* often occurs in high-density patches (Guimarães 2003) and *U. ornatrix* frequently move from one plant to another (Guimarães, pers. obs. 2003). Hence, although predation by ants was probably minimized by a behavioural response against ants, ants still deterred the *U. ornatrix* larvae.

The positions of the extrafloral nectaries and the phenological variation in ant attraction are probably the main factors that tune the ant defence as a seed predator deterrent mechanism rather than a general herbivore deterrent mechanism. EFN occur very close to flowers and unripe pods of *C. pallida*, thereby guaranteeing that ant activity and, consequently, deterrence are concentrated almost exclusively in racemes. This pattern was also observed for ants visiting EFN of *Caryocar brasiliense* (Caryocaraceae) (Oliveira 1997). In *C. pallida*, the extrafloral position of the nectaries implies that the defence of leaves, although it occurs, is probably a minor advantage of ant-guard defence. In addition to the position of EFN, plants also control the attraction of ants over time. In *C. pallida,* visitation by ants was higher in racemes with reproductive structures that were vulnerable to attack by *U. ornatrix* than in other racemes. In several plant species, EFN are active from the earliest stages of flower development until fruit ripening (Keeler 1980, 1981; Ganeshaiah & Shaanker 1988; Freitas *et al*. 2001; Falcão *et al*. 2003). Future studies should focus on potential trade-offs between defence of reproductive structures and pollination failure as a result of 'protection' of flowers against legitimate pollinators. In *C. pallida*, there are at least two non-mutually exclusive explanations for the termination of ant activity on racemes with ripe pods. First, since *U. ornatrix* do not attack ripe pods, the reduction in extrafloral nectar production may represent an economy of resources, especially if the eventual protection of vegetative parts promoted by EFN does not overcome the costs of nectar production. Second, at any given time, *C. pallida* have racemes in different phenological stages, and the end of nectar production on a raceme with ripe pods may shift the foraging pattern of ants on the plant, thereby enhancing the protection of vulnerable, reproductive structures. Future work should investigate the extent to which the phenological variation in ant-activity on extrafloral nectaries is tunable and its potential for maximizing plant defence against seed predators.

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