

Plant-sex-biased tritrophic interactions on dioecious willow

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Abstract. Plant sex effects on herbivores are well studied, but little is known about these effects on predators and predator–herbivore dynamics. Here we take a holistic approach to study, simultaneously, plant sex effects on herbivore and predator preference and performance, as well as population densities and predation pressure in the field. For dioecious *Salix cinerea* (grey willow) we found that male plants represented higher host plant quality than females for an omnivorous predator (*Anthocoris nemorum*, common flower bug), while host plant quality for its herbivorous prey (*Phratora vulgatissima*, blue willow beetle) was not sex-biased. The herbivore strongly preferred the host plant sex (female) that was suboptimal for the predator, which in turn followed its prey to female plants, leading to plant-sex-biased predation. These results provide new insight into the far-reaching effects of plant sex on insect communities, and open up novel opportunities for improving biocontrol of the herbivore in *Salix* short rotation coppice.

Key words: community genetics; dioecy; extended phenotype; genetic variation; herbivory; indirect effects; interaction modification; plant gender; sex-biased herbivory; tritrophic interactions.

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INTRODUCTION

Dioecious plants, which have separate male and female individuals, constitute popular model systems for studying plant effects on the surrounding insect community. Male and female plants are easy to identify in the field and differ in phenotypic characteristics that are of importance to insects. For example, female plants invest more in reproduction (flowers and seeds) than males (only flowers) (Obeso 2002); it has, therefore, been hypothesized that females invest more in defensive traits and less in vegetative growth compared to male plants (Lloyd and Webb 1977). These intersexual differences in

resource allocation have been confirmed for many, but not all investigated plant species (e.g., Bañuelos et al. 2004, Maldonado-López et al. 2014). Not surprisingly, intersexual differences have proved important for herbivores, and their identification has spurred a flurry of studies on sex-biased herbivory in dioecious plants over the last three decades (reviewed by Boecklen and Hoffman 1993, Ågren et al. 1999, Ashman 2002, Cornelissen and Stiling 2005, Avila-Sakar and Romanow 2012). These studies convincingly show that plant sex often controls herbivory and herbivore abundance from the bottom up, through intersexual differences in host plant quality, e.g., leaf size (Boecklen et al. 1990) and

chemical compounds (Bañuelos et al. 2004).

While many studies have examined sex-biased herbivory, it is striking that the effects of intersexual differences on predators and predation have been almost completely ignored. One noteworthy exception is Petry et al.'s study (2013) of multitrophic interactions on dioecious *Valeriana edulis*. They found 50% more aphid predators on female plants, driven by greater attractiveness of females, but the top-down effect on aphids was small. The paucity of studies is surprising given the emerging consensus that herbivore populations are often as strongly controlled by predators in a top-down manner as by host plant quality via bottom-up factors (e.g., Hairston et al. 1960, Oksanen et al. 1981, Halaj and Wise 2001, Terborgh et al. 2006). Dioecious plants express intersexual differences in several traits that are utilized by predators, not least nectar composition (Dötterl et al. 2014) and pollen availability. Differences in such plant-based resources can potentially affect both predator population dynamics (Ågren et al. 2012) and their willingness to consume animal food (Stenberg et al. 2011b). Further investigations of plant sex effects on predators and predator-prey interactions would thus be valuable.

Salix spp. (willows) have been important study species for sex-biased herbivory from the very start of these investigations (e.g., Danell et al. 1985, Boecklen et al. 1990, Danell et al. 1991, Hjältén 1992, Ahman 1997). In this study we use *Salix cinerea* L. (grey willow) to advance our understanding of plant sex effects on the behavior, performance, predation pressure, and population densities of both predators and herbivores in natural populations. By using naturally growing plants for our field studies, we avoid the scale problem associated with common garden experiments, where plant genotypes have been collected from a large geographic area and tend to overestimate plant effects at smaller scales (Tack et al. 2012). Another weakness with many previous studies is that they only estimate abundances and/or densities of arthropods without elucidating the underlying mechanisms. In order to investigate these underlying mechanisms it is important to conduct manipulative experiments and bioassays both in the field and in the laboratory.

The aim of this study was to elucidate how

plant sex shapes herbivore and predator population densities and predation rates in the field, and the mechanisms responsible for these altered patterns and processes. We hypothesized that male plants would increase the performance of the specialized herbivorous pest insect *Phratora vulgatissima* L. (Coleoptera: Chrysomelidae) and its omnivorous predator, *Anthocoris nemorum* L. (Heteroptera: Anthocoridae) when reared on a purely plant-based diet in controlled no-choice experiments in the laboratory. We further hypothesized that the insects would show behavioral adaptations to plant sex, navigating towards the superior plant sex in terms of host plant quality, prey availability, and predation risk. Finally, we hypothesized that the herbivore's preferred plant sex would support higher population densities and higher predation rates in the field.

METHODS

Study area and organisms

Salix cinerea L. (grey willow) is a perennial deciduous bush or small tree native to Sweden and very common in the study area. It flowers early in the spring and is thus an important food source for many flower-visiting insects at a time of year when few other plants are in flower. Male and female catkins are produced on separate individuals. *Salix cinerea* grows mainly in moist habitats like roadside ditches, meadows, and along water courses.

All experiments described in this article were performed using *Salix cinerea* bushes growing in roadside ditches along the county road between Märsta (59°16.555' N, 17°43.223' E) and Gåvsta (59°56.336' N, E 17°52.350' E), North of Stockholm, Sweden. Thirty male and 30 female plants were identified along this 51 km long transect. Because plants growing very close to each other can sometimes be of the same clone, we selected the experimental plants to maximize the distance between them. The mean distance between the plants was 850 m, but shorter distances were allowed between plants of opposite sexes because they could not be the same clone. We used the same 30 male and 30 female plant individuals for all experiments and observational studies.

All experiments and observational studies in this article were undertaken using naturally

growing *S. cinerea*; for the laboratory studies, detached floriferous twigs were used. The twigs used for laboratory experiments were removed using scissors: this is a method that has been shown not to induce defense responses in *S. cinerea* (Dalin and Björkman 2003). For all field experiments, one bush was considered to represent one replicate. For laboratory experiments, several twigs from all 60 bushes were collected and placed in buckets containing either male or female twigs, but without keeping track of individuals.

Phratora vulgatissima L. (Coleoptera: Chrysomelidae, blue willow beetle) is an important herbivore on several willow species in Northern Europe. It is native to the study area and is considered the most important pest insect on commercial short rotation *Salix* coppices (Björkman et al. 2000). *Phratora vulgatissima* is normally univoltine in the study area (Dalin 2011), but can produce a second generation during favorable conditions (own observations). It overwinters as an adult, and emerges in late April or early May, it then lays eggs on willow leaves in May and June. The eggs hatch after about 10 days, and the larvae feed on the *Salix* leaves until pupation, which takes place in the soil. The adult beetles used for the laboratory experiments in this article came from a laboratory culture fed with a mix of vegetative *Salix* plants of both sexes.

Anthocoris nemorum L. (Hemiptera: Anthocoridae, common flower bug) is an omnivorous predatory bug that is common in, and native to, Sweden. It overwinters as an adult, and produces several overlapping generations each year. It emerges early in the spring, often several weeks before its primary prey species (*P. vulgatissima*). As indicated by its vernacular name, it is often seen foraging on catkins, but it can also sustain itself on vegetative willow alone, without access to flowers or animal food (Stenberg et al. 2010, 2011a). However, its fitness is significantly increased when provided with animal food (Stenberg et al. 2010, 2011a). Its most important animal food source is *P. vulgatissima* eggs and small larvae. The *A. nemorum* individuals used in the laboratory experiments in this research were collected from wild nettles (*Urtica dioica*); this species was selected because it is unrelated to *Salix*.

Insect performance on male and female plants

Controlled no-choice experiments were performed in climate chambers in order to investigate the performance of *P. vulgatissima* and *A. nemorum* on the two plant sexes. These experiments were undertaken in a strictly biotrophic setting such that *P. vulgatissima* performance was tested without predators, and *A. nemorum* performance was tested in the absence of animal food, i.e., without beetles.

Anthocoris nemorum performance.—The performance of *A. nemorum* on male and female *S. cinerea* was estimated by measuring development time (from hatching to adult) and adult body length of *A. nemorum* reared individually on floriferous detached 20 cm long twigs in the climate chamber setup described in *Phratora vulgatissima* performance. Newly hatched *A. nemorum* for the experiment were obtained by collecting adult individuals from wild nettles (*Urtica dioica*) and letting them lay eggs on green beans (*Phaseolus vulgaris*) in the laboratory. First instar individuals (<48 h old) were randomly selected for the experiment.

The rearing containers for the *A. nemorum* performance experiments were 20 cm high and 9 cm wide. One male or one female floral twig was placed vertically in each container. The lower 7 cm of the containers were filled with water; a seal with a drilled hole for the twig to be inserted was placed over the water surface to prevent insects from entering the water. The insects were released individually (one insect per container) to the upper part of each container and had access to the upper parts of the twigs, including leaves and catkins. The twigs were replaced as necessary, such that the insects always had access to fresh twigs. The containers were inspected every day until all individuals reached the adult stage, and the development time (days to adult stage) was recorded. The body length of the adult individuals was measured using a binocular microscope. We examined 29 insects per plant sex.

Phratora vulgatissima performance.—Lehrman et al. (2012) evaluated several different methods for estimating *Salix* food host plant quality for *P. vulgatissima* and found that the number of eggs laid on detached leaves in no-choice experiments is the most robust measure of host plant quality. Here we used Lehrman's protocol to estimate *P.*

vulgatissima performance on detached leaves of male and female *S. cinerea*. In total, 48 mated adult *P. vulgatissima* females were placed individually in plastic rearing containers (26 × 67 mm, perforated lid). All containers were placed in a climate chamber (18.5°C, 80% RH, 16:8h light regime); half (24) of the beetles were fed detached female leaves (one new leaf was provided every second day), while the other half were fed detached male leaves. The number of eggs laid was counted every second day when the leaves were exchanged. The experiment was terminated after eight days, following the recommendation of Lehrman et al. (2012).

Insect preference for male or female plants

Choice experiments were performed with *P. vulgatissima* and *A. nemorum*, separately, to determine whether they have any preference for male or female *S. cinerea*. This was done by putting a randomly paired set of one male twig and one female twig into an experimental arena. The two twigs did not have leaf contact with each other, and each twig was only used once. The containers described in *Anthocoris nemorum preference* (above) were used, with the difference that the seals had two drilled holes (5 cm apart), one for each of the two twigs. In each case, a single insect was inserted into the arena and after 30 minutes the insect's whereabouts (male or female twig) were noted. Fifty replicates were used for each insect species. Adult female *P. vulgatissima* for this experiment were obtained from the laboratory culture; adult *A. nemorum* were collected from nettles (*Urtica dioica*) in the field, and were kept on green beans (*Phalesolus vulgaris*) in the lab for 48 hours prior to the experiment. The sex of the *A. nemorum* individuals was not determined.

Insect densities on male and female plants in the field

The densities of the herbivore *P. vulgatissima*, and the omnivorous predator *A. nemorum* on the two plant sexes were recorded on three occasions during summer 2011 (May 28, June 19, June 27). The male flowers have fallen in June, whereas female flowers remain to produce seeds. The three sampling occasions were included to investigate whether insect density patterns changed over the summer. A total of 22 male

and 22 female bushes were randomly selected for this study. Insects were collected by knockdown sampling (sensu Björkman et al. 2003) from a 35-cm section at the top of a shoot. In addition, *A. nemorum* densities were recorded on 13 male and 16 female plants on June 7, 2013 using a sweep net. Predators were collected by sweeping the net through the foliage five times per plant. Only adult insects were included in the surveys.

Egg predation on male and female plants in the field

The predation pressure imposed by *A. nemorum* on *P. vulgatissima* eggs on the two plant sexes was studied in a field experiment which started on June 19, 2011. Newly laid egg clutches (<24 h old) with 10 eggs each were collected from the laboratory culture. Twenty-one male and 21 female *S. cinerea* plants were randomly selected for this study. One egg clutch with 10 eggs was attached to each plant using insect pins. The number of eggs consumed by predators was counted every day until eggs started to hatch, which happened on the seventh day of the experiment. Eggs consumed by *A. nemorum* are easily distinguished from intact and hatched eggs, as the damaged egg shell and part of its contents remain.

Statistical analyses

To test for *S. cinerea* sex effects on body length and time to adulthood in the *Anthocoris nemorum* performance experiment, linear models with body length and development time as response variables and sex as a treatment were tested using analyses of variance (ANOVAs). Similarly, to test for *S. cinerea* sex effects on *P. vulgatissima* performance, an ANOVA was used to test a linear model with *P. vulgatissima* eggs as the response variable and sex as a treatment.

Chi-square tests were used to determine whether there were differences in insect preferences towards either plant sex in the cafeteria experiment. To test for plant sex-related differences in field abundances for each of the insect species in 2011, generalized linear mixed models (GLMMs) with quasi-Poisson distributions and log-links were created using the glmer function in the lme4 package for R. Models with insect abundance as a response variable, *S. cinerea* sex as a factor and plant individual as a random variable

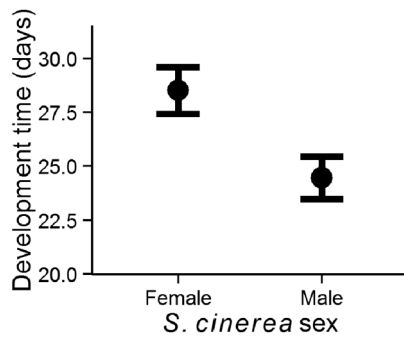


Fig. 1. Development time (days from first instar to adult, means \pm SE) for *Anthocoris nemorum* on female and male twigs of *Salix cinerea*. N = 29 insect individuals per plant sex.

were compared to corresponding models with constants instead of *S. cinerea* sex as a factor in likelihood ratio tests. For the 2013 *A. nemorum* abundance data, a GLM with a Poisson distribution and log-link was used, with abundance as a response variable and *S. cinerea* sex as a factor. To examine *S. cinerea* sex effects on egg predation, a GLMM with a binomial error family and log-link was used. To test for differences in egg survival over time, a generalized linear mixed model with egg survival as a response variable was constructed. Time and *S. cinerea* sex were used as explanatory independent variables with an interaction term, and plant individual was used as a random factor. The model was chosen after comparison to corresponding models with the interaction and variables removed. These comparisons were made using likelihood ratio tests. All analyses were performed using R 2.15.3 (R Development Core Team 2013). Figures were created using the basic and ggplot2 packages for R.

RESULTS

Insect performance on male and female plants

Anthocoris nemorum.—The development time from egg hatching to the adult stage for *A. nemorum* was about four days shorter for those reared on male twigs than those reared on female twigs (df = 1, $F = 7.52$, $P = 0.008$; Fig. 1). Body lengths at the adult stage did not differ between insects reared on male and female twigs (df = 1, $F = 0.974$, $P = 0.328$).

Phratora vulgatissima.—The numbers of eggs

Table 1. Individuals of the herbivore *Phratora vulgatissima* and the omnivore *Anthocoris nemorum* preferring male or female *Salix cinerea* plants in two-choice cafeteria experiments in the laboratory. Chi-square tests were used to test for differences in insect preferences.

Species	Male	Female	df	χ^2	P
<i>P. vulgatissima</i>	12	33	1	9.8	0.002
<i>A. nemorum</i>	18	32	1	3.92	0.047

laid by *P. vulgatissima* did not differ between male (16.2 ± 1.7) and female (16.1 ± 1.9) plants (df = 10.977, $t = 0.866$, $P = 0.404$), indicating that the host plant quality of *S. cinerea* as a food for this herbivore does not differ between plant sexes.

Insect preferences for male or female plants

Both *P. vulgatissima* and *A. nemorum* strongly and significantly preferred female twigs over male twigs in the cafeteria experiment (df = 1, $\chi^2 = 9.8$, $P = 0.002$ and df = 1, $\chi^2 = 3.92$, $P = 0.047$; Table 1).

Insect densities on male and female plants in the field

The abundances of *P. vulgatissima* were higher on female than male *S. cinerea* in 2011 (df = 1, $\chi^2 = 13.773$, $P < 0.001$; Fig. 2). The abundances differed between sampling occasions (df = 2, $\chi^2 = 13.559$, $P = 0.001$), being highest on the first occasion. Abundances of *A. nemorum* were significantly higher on female than male *S. cinerea* plants in 2011 (df = 1, $\chi^2 = 10.1347$, $P = 0.001$; Fig. 2) and 2013 (df = 1, $F = 5.0259$, $P = 0.033$; Fig. 2). There was no difference in *A. nemorum* abundances between the sampling occasions in 2011 (df = 2, $P = 0.271$; Fig. 2).

Predation in the field

There was an interaction between time and plant sex effects on *P. vulgatissima* egg survival (df = 1, $\chi^2 = 71.062$, $P < 0.001$), with the surviving *P. vulgatissima* eggs decreasing more rapidly on female than male twigs (Fig. 3, Table 2). At the end of the experiment the abundance of surviving eggs was, on average, four times higher on male twigs compared to female twigs.

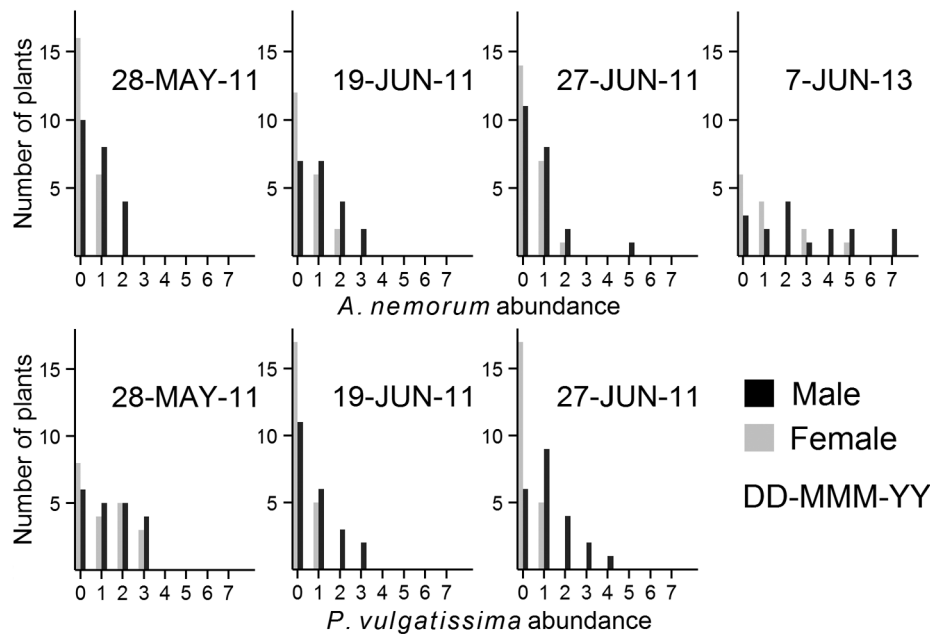


Fig. 2. Frequencies of observed field abundances (individuals scored per plant using knock-down sampling) of the omnivorous predator *Anthocoris nemorum* and its main herbivore prey *Phratora vulgatissima* on wild male and female *Salix cinerea* plants. N = 22 male and 22 female plants for 2011, and 13 male and 16 female plants for 2013.

DISCUSSION

We found that plant sex affected an omnivorous predator, its main herbivorous prey, and their interaction on *Salix cinerea*. In line with our

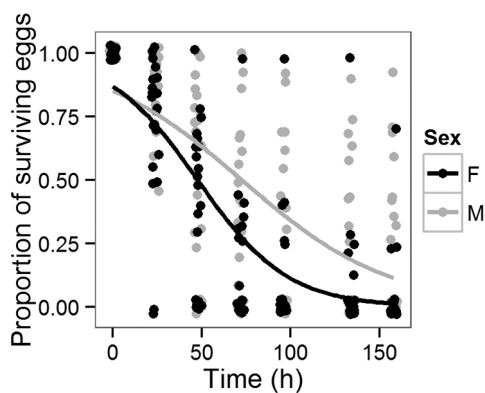


Fig. 3. Data distribution of the proportion of surviving *Phratora vulgatissima* eggs on female (black) and male (grey) *Salix cinerea* plants recorded on seven occasions. The curves denote estimated logistic regressions of proportional egg survival over time on female and male plants. A jitter function separates overlapping data points. N = 21 male and 21 female plants.

hypothesis, female plants represented lower host plant quality for the omnivorous predator than male plants. The four-day difference in development time between predators on male and female plants is remarkably high, and results in two types of fitness-reducing costs. First, slow growth is likely to result in greater exposure to natural enemies and a consequent increase in mortality (Benrey and Denno 1997). Second, in this omnivorous predator (*A. nemorum*) such reductions in host plant quality also directly translate into reduced reproduction and population growth, all else being equal (Stenberg et al. 2010, 2011a, Ågren et al. 2012). Decreased

Table 2. Effects of time and plant sex on survival of *Phratora vulgatissima* eggs in the field. The eggs were experimentally attached to male or female plants and monitored for seven days. All (100%) of the egg mortality was caused by predation by omnivorous bugs (mainly *Anthocoris nemorum*).

Variable	χ^2	df	P
Time	1044.200	1	<0.001
Plant sex	3.1975	1	0.073
Time \times plant sex	71.0619	1	<0.001

predator performance and growth should be good news for the herbivore. Indeed, we found that the herbivore showed a behavioral preference for female plants, and had higher densities on females in the field, despite the fact that host plant quality of male and female plants was identical for the herbivore. Although using female plants as hosts does not provide the herbivore with enemy-free space, it forces the omnivorous predator to interact within a suboptimal arena where its growth will be limited.

The omnivorous predator too showed a behavioral preference for female plants. The preference for female plants suggests that the omnivorous predator follows its most important food source, namely the herbivore, which is more abundant on female plants. Previous studies have shown that availability of animal food increases the predator's fitness more than high host plant quality, although both are needed for maximal predator growth (Stenberg et al. 2010, Ågren et al. 2012).

In accordance with the preference for female plants, we found that, in the field, the predator is more abundant on females despite the male plants providing higher host plant quality. The higher predator abundance on female plants is probably linked to its preference for females (migration to female plants), but a higher population growth rate due to higher prey availability (as shown by Stenberg et al. (2011a)) could also be important and the current study cannot separate these two factors.

Interestingly, the predation pressure on the herbivore eggs in the field was much higher on female than male plants. Thus, in a system where most predators and herbivores dwell on female plants it can actually pay dividends for individual herbivores to go against the current trend and oviposit on male plants, despite this being the predator's optimal host plant. One can imagine that natural selection for utilizing male plants, at least temporarily, could drive the herbivores to male plants, but if the predators tend to follow their prey the predation pressure on male plants would quickly increase, and the herbivores would soon experience the disadvantage of being on the predator's "home ground". An evolutionary arms race (Van Valen 1973) between the predator and the herbivore may eventually drive repeated host shifts between the two plant sexes,

but we expect the pendulum to move quickly away from male plants. If an evolutionary arms race exists in this system one should find geographic variation (Thompson 2005) in host plant preferences of the herbivore and the predator; future studies should investigate this possibility.

The results of this study show that the effects of plant sex on insects are not limited to bi trophic interactions, but involve ecological interactions at higher trophic levels. Petry et al. (2013) also found that insect predators were more abundant on female plants in a system based on *Valeriana edulis*. The present study is unique in that it shows not only predictable plant sex effects on the densities of herbivores and predators, but also on the predation pressure on the herbivores, as well as a mechanism involving plant effects on predator performance as a candidate key driver behind the observed patterns.

In parallel with the advance of our basic ecological knowledge of plant sex effects on herbivores and predators, it is also clear that the new knowledge can be utilized in genotype selection and plant breeding for improved biocontrol in short rotation coppice. Short rotation willow coppices are often composed of a single *Salix* clone, implying that the entire plantation has one single plant sex only (A. Henriksson, *personal communication*). Currently most plantations in Europe are all-female (A. Henriksson, *personal communication*; J. A. Stenberg, *personal observations*). The herbivore included in this study constitutes the major pest insect in plantations, reducing stem wood production by up to 40% (Björkman et al. 2000). Recent findings show that the predator exercises a weaker top-down effect in plantations than in natural stands (Dalin et al. 2009). The results of the current study raise the question of whether the weak biocontrol in plantations is due to the fact that female *Salix* varieties are used. Future studies should investigate whether male plantations with omnivorous predators would experience less herbivory and higher cropping security than female plantations. The emerging holistic understanding of plant sex effects on insect communities foreshadows a new platform for designing integrated and environmentally friendly management of pests and diseases in dioecious crops.

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