

WHERE DID ALL THE OFFSPRING GO?

POLLINATION AND PRE-DISPERSAL

SEED PREDATION IN A

CARNIVOROUS PLANT

by

Emma Brilleslyper

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Project Approved:

Supervising Professor: John Horner, Ph.D.

Department of Biology

Amanda Hale, Ph.D.

Department of Biology

Dean Williams, Ph.D.

Department of Biology

ABSTRACT

Carnivorous plants are threatened by factors such as habitat destruction, so it is important to understand the factors affecting these populations. The size and expansion of plant populations is dependent in part on the production of offspring (seeds). However, the number of seeds produced can be influenced by pollination levels and, once produced, seeds can be lost to seed predation. We analyzed the proportion of ovules (immature seeds) fertilized per ovary and the proportion of seeds lost to pre-dispersal seed predation in two populations of the carnivorous plant *Sarracenia alata*. Mature ovaries were collected from flowers in Middle Branch Bog in Kisatchie National Forest, Louisiana in 2008 and from a bog in Leon County, TX in 2015, 2016, and 2019. The average proportion of ovules fertilized in 2019 (0.178 ± 0.066 ; mean \pm SE) was significantly lower ($P < 0.001$) than that in the other years sampled (0.779 ± 0.048 , 0.736 ± 0.029 , 0.694 ± 0.066 , for the 2008, 2015, and 2016 samples, respectively). The average percent of seeds consumed by *Endothenia daekaena* across years in the Leon County site was $27.06\% \pm 0.094$ and did not differ significantly ($P > 0.05$) among years. Combined, these data demonstrate a significant loss of reproductive effort due to pollen limitation and pre-dispersal seed predation.

Introduction

The size and expansion of plant populations is dependent in part on the production of offspring. Pollination is essential to the reproduction of flowering plant species. Pollinator populations, particularly bees, have been declining for years, and subsequent pollen limitation has had an impact on seed production (Baron et. al 2017). Once produced, seeds can be lost to seed predation. Seed predation directly impacts the fitness of the species and may cause a change in ecosystem diversity. It causes a direct reduction in fitness as individuals that lose seeds to predation, whether pre-or post-dispersal, have fewer viable offspring (Larios et al. 2017). Predation patterns can vary in time and place and understanding seed predation in environments where plants are threatened will be helpful in our efforts to conserve them (Kolb et al. 2007). Pre-dispersal seed predation is common amongst many plant species, and it could lead to losses of offspring greater than 50% (Boieiro et al. 2010; Kolb et al. 2007). Carnivorous plants are an interesting model for examining ovule fertilization and seed predation due to their unique adaptations and dwindling populations. There are currently few studies on ovule fertilization and no published studies on pre-dispersal seed predation in carnivorous plants.

Carnivorous plants often live in nutrient-poor bog environments and they capture and digest insects as an adaptation to supplement the nutrients they can acquire from the soil. They benefit from insect capture, as demonstrated by increased growth, earlier flowering, and increased seed production (Jürgens et al. 2012). Nutrient-poor environments present many challenges for plants, and seed predation further complicates those challenges. For example, reproduction requires considerable resources. Plants in nutrient-poor environments that invest in reproduction expend many of their limited resources on this activity. Thus, seed predation in such species represents a significant loss of scarce resources.

We examined seed production and pre-dispersal seed predation in the carnivorous plant *Sarracenia alata*. *Sarracenia alata* depends on pollinators for reproduction, but pollinators are experiencing global decline (Baron et. al. 2017). As pollinators are declining, the impact of pre-dispersal seed predation may be further exacerbated. *Sarracenia alata* occurs in wet pine savannas, a habitat which is increasingly threatened (Fig. 1). This makes *Sarracenia alata* a useful model to investigate seed predator-plant interactions.

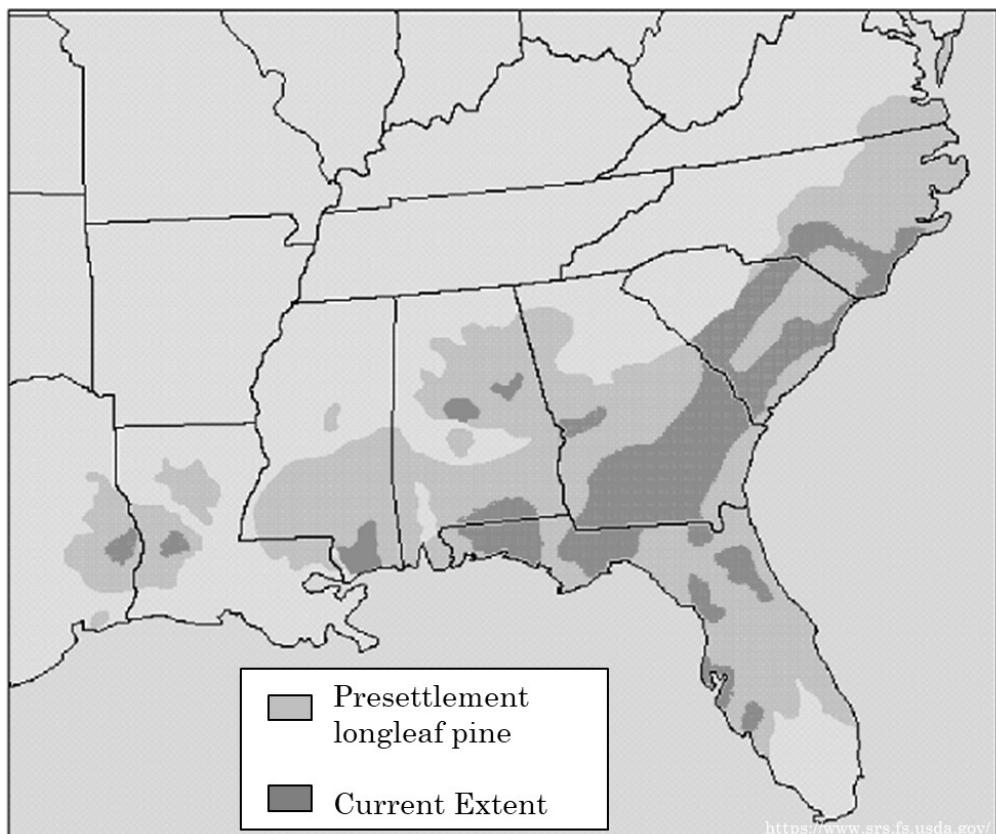


Figure 1. Current and historic distribution of wet pine savannas.

In order to address the issues of seed production and pre-dispersal seed predation, I examined two main questions. First, I examined how pollination (as a proportion of ovules

fertilized) varied among years. Secondly, I looked at variation in pre-dispersal seed predation among years.

Methods

Study organisms:

Sarracenia alata is a carnivorous, perennial herb that grows in bog environments in the southeastern United States from Alabama to Texas along the coast of the Gulf of Mexico (Horner et al. 2014; Figure 1). It produces a single flower at the beginning of the growing season and then produces pitchers formed from modified leaves (Fig. 2). Flowers are primarily pollinated by bees of the genus *Bombus* in late March or early April and are subject to pre-dispersal seed predation by *Endothenia*.

Bombus impatiens is the dominant pollinator of *S. alata* in our study sites (J. Horner, personal communication). *Bombus impatiens* is a generalist pollinator that visits many of the flowering plants at our study sites (personal observation).

We have tentatively identified the seed predator in our samples as *E. daekaena*. *Endothenia daekaena* (Lepidoptera: Olethreutidae) lays eggs on flower bracts of *Sarracenia purpurea* around July 1. The newly hatched larvae then bore into the ovary base to reach the seeds. First instar larvae enter seeds and consume them from within, and instars 2-5 consume seeds from the outside. Instars 4 and 5 leave the ovary to overwinter in the flower stalk. Pupation takes place in late May with adults emerging through the remaining epidermis (Hilton 1982).



Figure 2. *Sarracenia alata* flower (L) and pitcher (R)

Study Sites:

Ovaries for this study were collected from two study sites. Ovaries were collected from Middle Branch Bog in Kisatchie National Forest, Louisiana ($\sim 31^{\circ} 30' N, 93^{\circ} 4' W$) in 2008. Flowers were collected from a bog in Leon County, Texas ($\sim 31^{\circ} 18' N, 95^{\circ} 59' W$) in the fall of 2015, 2017, and 2019.

Laboratory methods:

Seed Assessment and Counting:

For each ovary, we used a series of sieves to sort unfertilized ovules from fertilized seeds based on size. Each seed/ovule was then examined microscopically and sorted into one of three groups; unfertilized ovules, seeds which had their contents consumed, and intact seeds. Masses were obtained for the seeds/ovules in each group from each ovary. Initially, each group was counted by hand.

We then developed a method to count seeds using a flatbed scanner and ImageJ software. Seeds were placed directly on the scanner bed, taking care to avoid clumps so as to improve the accuracy of the count. A white paper was placed behind the seeds to assist in image formation and to prevent the seeds from shifting. The seeds were scanned at a resolution of 200 pixels per inch (ppi). The scanned image was imported to ImageJ and converted to an 8-bit, black-and-white file. Each particle counted was outlined by the software in order to visually confirm that the software was not incorrectly counting seeds that were close together. Hand counts were compared to counts taken using the scanner and ImageJ. Once it was determined that there was no significant difference in the values obtained with the two methods (Fig. 3), the scanner method was used exclusively to count seeds. A total of 16,960 seeds were counted from 35 ovaries.

Ovule Counting

Because ovules are smaller than seeds, the method for ovule counting was refined in order to avoid accidentally including debris in ovule counts. Ovules were scanned separately from fertilized seeds at a higher resolution (600ppi). A random sample of ovules from 10 scans were processed to obtain a range for area and circularity of the ovules. Based on these measurements, the area for processing each scan was set to 0.02-0.4mm², and circularity was set to 0.5-0.98. The data obtained from counting particles using the refined area and circularity was compared to data obtained when the scan was taken at the same resolution with no processing restrictions. Because it was determined that the refined method (with area and circularity restrictions) consistently included fewer debris particles than the original method, all ovule scans were processed using the refined method. A total of 9198 ovules were counted from 35 ovaries.

Statistical Analysis

The proportion of ovules fertilized and the proportion of seeds consumed by *E. daekaena* were compared across years using separate analyses of variance (ANOVA) on arcsine-transformed data. Differences among years were determined by Tukey's multiple range test. Because samples were collected from the population in Kisatchie National Forest in a single year, site was not included as a factor in the statistical analysis. Statistical significance was inferred at $\alpha = 0.05$ for all analyses. All statistics were performed in Excel 2016. Means are presented as \pm SE (standard error) throughout the manuscript.

Results

Seed Counting Method Development

Seeds from 16 ovaries from the Leon Co., TX site collected in 2015 were counted by hand and were also scanned and processed using ImageJ. Because of the strong correspondence between counts performed by hand and by ImageJ (Figure 3), ImageJ processing was used to obtain all seed counts for the remainder of the samples.

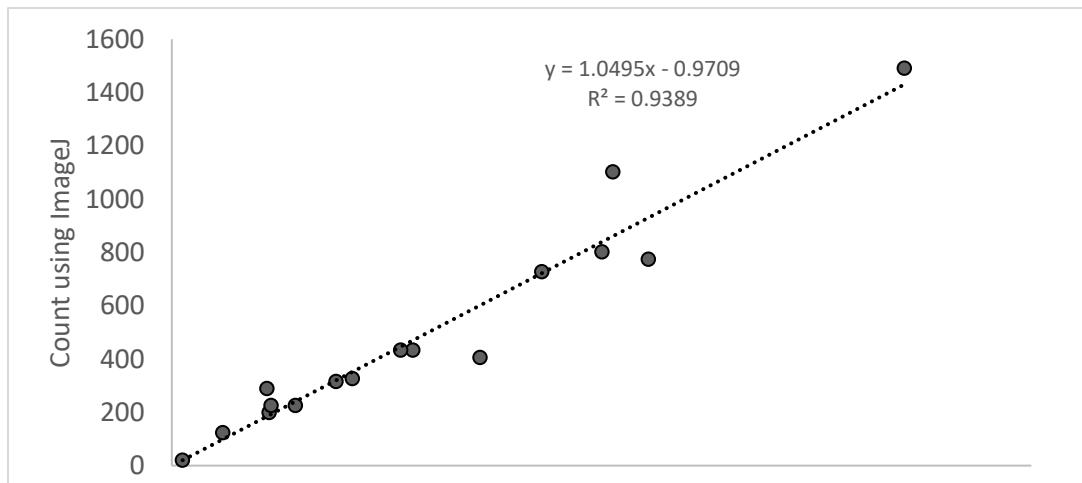


Figure 3. Correlation between seed counts determined by hand and those determined by scanning and analysis by ImageJ software

Ovule Counting Method Development

Data from the unrestricted ImageJ analysis were compared to those using the size and circularity restrictions. The restricted analysis eliminated more debris (dust and ovary wall particles), as confirmed by microscopic analysis (Figure 4; K. Kang, personal observation). Ovules from the rest of the ovaries were counted using the restricted method.

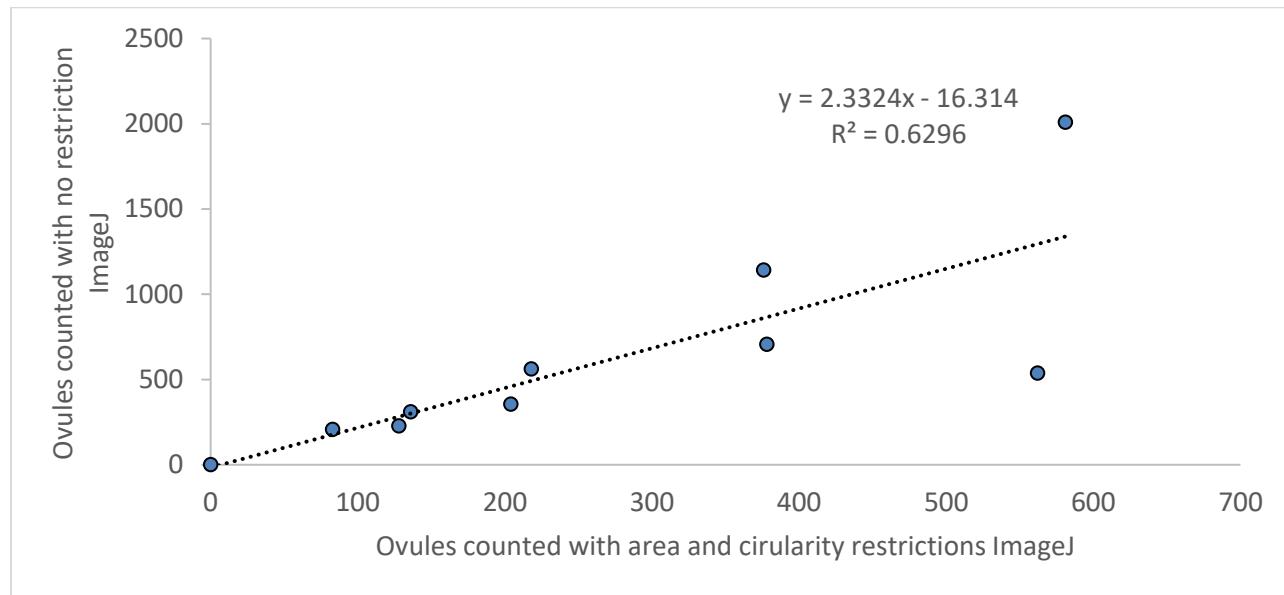


Figure 4. Correlation between ovule counts determined by scanning and analysis with ImageJ with no particle size restrictions and those determined by scanning and analysis by ImageJ software with circularity and area restrictions applied for particle processing. Circularity and area restrictions eliminated debris particles.

Proportion of Ovules Fertilized

The average proportion of ovules fertilized in 2019 was significantly lower than that in the other years sampled (Table 1, Fig. 5).

Table 1. Summary of analysis of variance (ANOVA) for the effects of year on the proportion of ovules fertilized for all years and both sites.

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>F</i>	<i>P</i>
Between Years	2.9081	3	22.34	6.78E-08
Error	1.3449	31		

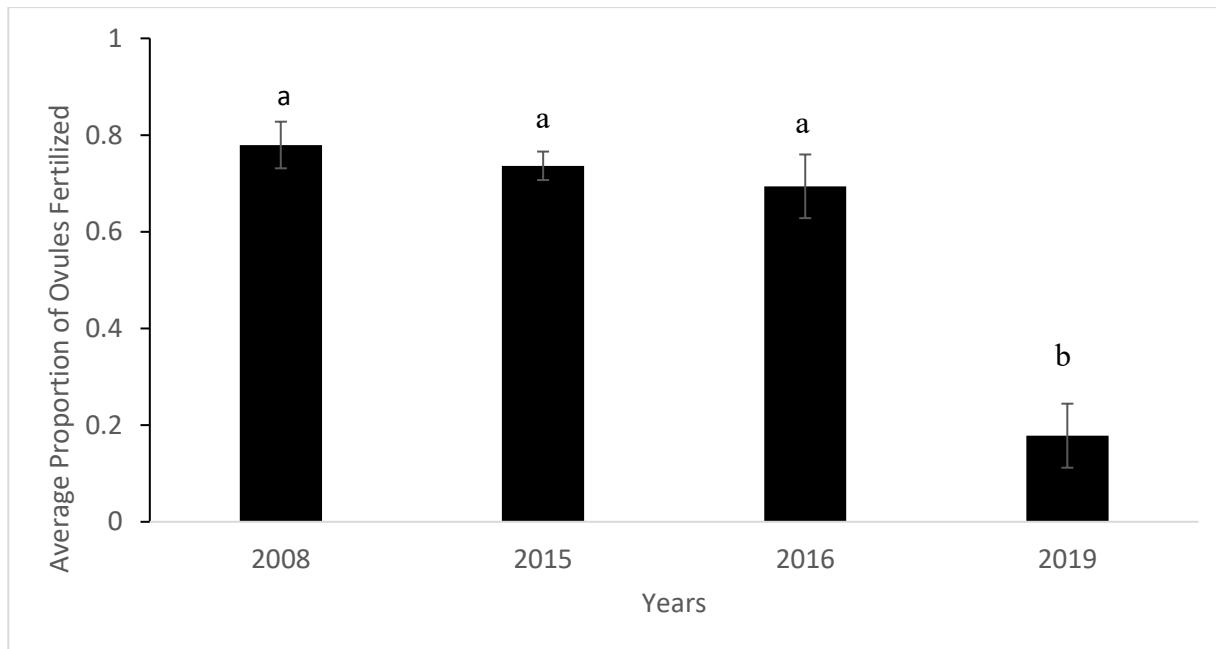


Figure 5. Average proportion of ovules fertilized for all years and both sites. Whiskers are \pm 1 SE

Extent of seed predation by *E. daekaena*

The average percent of fertilized ovules consumed by *E. daekaena* across all years was 20.33% \pm 9.3. The average percent of seeds consumed by *E. daekaena* across years in the Leon County site

was $27.06\% \pm 0.094$ and did not differ significantly ($F_{2(2)22} = 0.0077$, $P > 0.05$) among years. The average percent of fertilized ovules consumed in the population from the Kisatchie site (from 2008) was $0.167\% \pm 0.1$; only two of the eleven ovaries obtained from this location showed signs of pre-dispersal seed predation. When data from all years at both sites were combined, the quantity of pre-dispersal seed predation in 2008 (Kisatchie) was significantly lower than that from Leon County (Table 2, Fig. 6).

Table 2. Summary of the analysis of variance of the average proportion of seeds consumed by *E. daekaena* annually during all years at both sites.

ANOVA

<i>Source of Variation</i>	SS	df	F	P
Between Groups	1.6293	3	5.13	0.005
Error	3.4936	33		

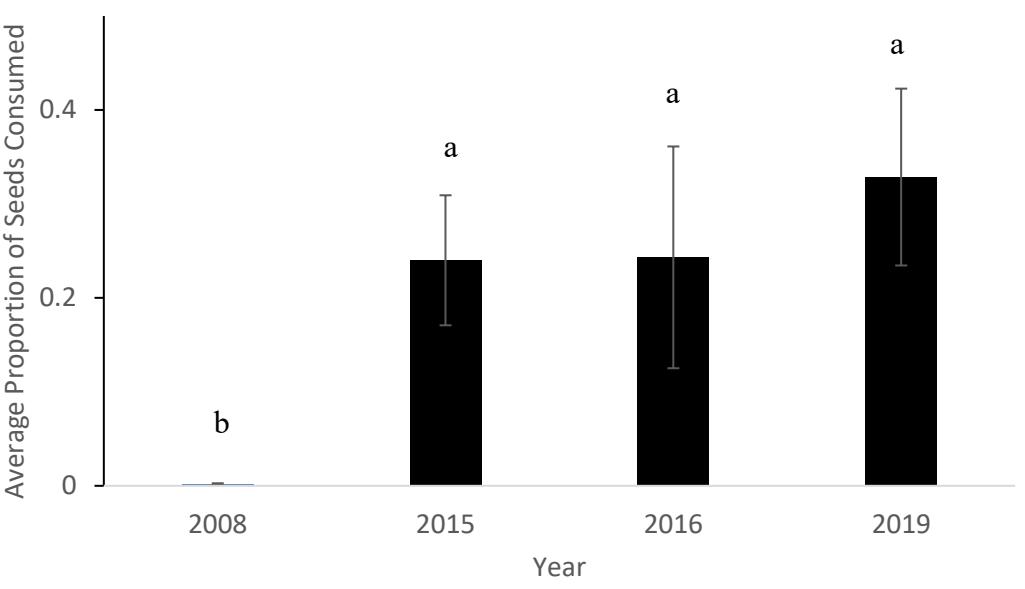


Figure 6. Average proportion of seeds consumed by *E. daekaena* annually at all sites. Whiskers are ± 1 SE.

Discussion

Pollinator activity and pre-dispersal seed predation both contributed to a loss of reproductive effort in this study. In 2019, when there was low pollinator activity (J. Horner and K. Kang, personal communication), the proportion of ovules fertilized was only 0.178. This is significantly lower than the average proportion of ovules fertilized from all other years, implying that pollen limitation can affect the production of viable offspring on a temporal basis. On average across the other years (years with “normal” pollinator activity), the proportion of ovules fertilized was 0.736.

There was evidence of pre-dispersal seed predation in all years in both locations, although predation was significantly lower among the ovaries taken from Kisatchie for reasons unknown. The Kisatchie site had an average of 0.167% of fertilized seeds lost to pre-dispersal seed predation. Among ovaries taken from the Leon County site, predation accounted for between 24% (2015) and 32.8% (2019) of fertilized ovules being lost. Although differences in populations of *E. daekaena* between sites probably was a factor in the significant difference in seed predation observed between the Kisatchie site (2008) and the Leon county site (2015, 2016, and 2019), we cannot exclude other factors (e.g., differences in *E. daekaena* activity in 2008). As other studies have shown, pre-dispersal seed predation may be responsible for losses of up to 50% (Boieiro et al. 2010; Kolb et al. 2007). Thus, levels of pre-dispersal seed predation observed in *S. alata* are significant but fall within the range reported in other studies.

The combination of loss due to a lack of fertilization and loss due to pre-dispersal predation gives a loss of 22.3% of potential offspring for the Kisatchie site, and an average loss of 53.3% of potential offspring for the Leon County site. Approximately 75% of potential seeds

are fertilized across all years and of those approximately 30% are lost to pre-dispersal seed predation. Even in a low fertilization year like 2019, seeds were lost to *E. daekaena*, resulting in a significant combined loss. If the size of the seed predator population remained relatively constant, we would expect the number of seeds consumed to remain constant also, and thus the proportion of seeds consumed in 2019 to be greater. It is unclear why this is not the case, but it may be due to an inability to account for ovules or seeds completely consumed by *E. daekaena* using our methods.

In addition to losses of reproductive effort due to pollen limitation and pre-dispersal seed predation, *S. alata* loses offspring to floral herbivory. *Exyra semicrocea* is a specialized herbivore that feeds on both *S. alata* traps (Carmickle & Horner 2019) and flowers (Winer 2019). *Exyra semicrocea* may consume up to 50% of ovary mass, which represents a substantial loss of ovules even if it is assumed that some of the lost mass was ovary wall. Floral damage by *E. semicrocea* may lead to reduced pollinator visitation and reduced pollen transport in addition to causing a direct loss of seeds and flower parts (Winer 2019).

In conclusion, we found significant annual variation in pollination (fertilization of available ovules) and in losses of offspring to pre-dispersal seed predation in *S. alata*. This understanding of reproductive losses in *S. alata* contributes to our knowledge of the intricate ecological interactions in this fascinating carnivorous plant and may be used in conservation efforts.

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