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Elevated carbon dioxide increases nectar production in *Epilobium angustifolium* L.

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Abstract Effects of elevated CO_2 and nutrient availability on nectar production and onset of flowering in five different seed families (genotypes) of *Epilobium angustifolium* were investigated in a greenhouse experiment. Elevated CO_2 significantly increased nectar production per day (+51%, $p < 0.01$), total sugar per flower (+41%, $p < 0.05$), amino acid concentration (+65%, $p < 0.05$) and total amino acids per flower (+192%, $p < 0.001$). All other parameters tested, i.e., nectar sugar concentration, proportion of glucose/fructose and proportion of sucrose/(glucose + fructose), were not significantly affected by elevated CO_2 and/or fertilization. However, elevated CO_2 caused a marginally significant trend for earlier flowering in highly fertilized plants. No significant family × CO_2 interaction was found in any of the tested parameters, but the response in nectar production varied considerably among seed families (+10 to +104%) and was significantly positive in two of the five seed families investigated. Our results are not consistent with earlier studies on effects of elevated CO_2 on nectar production and flowering phenology in other plant species. It seems, on the other hand, that CO_2 effects on nectar production are specific to species and genotype. Hence, no general conclusions about effects of elevated CO_2 on these floral traits can be drawn at present, but it must be cautioned that elevated CO_2 might not only increase floral rewards as in *E. angustifolium*, but might also lead to shifts or even disruptions in fine-tuned plant–pollinator interactions.

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Introduction

Since the early days in plant pollination research, nectar has been found to be an essential reward for pollinators (Sprengel 1793). In fact, nectar is the most frequent and widespread floral reward besides pollen (Faegri and van der Pijl 1979; Baker and Baker 1982; Simpson and Neff 1983; Proctor et al. 1996). The thorough investigations of Baker and Baker (1973, 1980, 1982, 1983) on the chemical composition of nectar showed characteristic patterns in nectar sugar and amino acid composition and concentration, and suggest that both nectar quality and quantity are subjected to strong selection by different groups of pollinators. While nectar characteristics can differ substantially between different plant species, they are relatively constant within species (Baker and Baker 1977; Lanza et al. 1995), although some intraspecific variation due to evaporation and aging of flowers has been reported (Corbet et al. 1979; Kradolfer and Erhardt 1995; Lanza et al. 1995; Hahn and Gzik 1998; Gardener and Gillman 2001a). Interspecific differences in nectar properties are mainly explained by differences in physiological demands and feeding properties of different pollinators (Baker and Baker 1982, 1983). However, differences in the amino acid composition of different nectar types, aside from affecting the taste of nectar (Gardener and Gillman 2002), still await a convincing explanation (Baker and Baker 1980).

During the last few years, rising concern about the expected increase in atmospheric CO_2 and the associated climatic changes has led to a substantial amount of research investigating effects of elevated CO_2 on plant physiology, plant–herbivore interactions and plant communities (Woodward et al. 1991; Koch and Mooney 1996; Körner and Bazzaz 1996; Walker et al. 1999; Körner 2000, 2002; Goverde and Erhardt 2002). How-

ever, there are only few studies investigating effects of elevated CO_2 on floral traits such as pollen germination, flowering phenology and nectar production (Osborne et al. 1997; Lake and Hughes 1999; Lavigne et al. 1999; Davis 2003). This is—in our view—a considerable void since the great amount of knowledge on plant–pollinator interactions shows complex and fine-tuned coevolved patterns (Faegri and van der Pijl 1979; Baker and Baker 1980; Armbruster 1996; Proctor et al. 1996; Wilson and Thomson 1996), which could be stressed or even disrupted by changes in availability and composition of floral rewards. Furthermore, the few available studies revealed rather contradictory and inconsistent responses in phenology and nectar production of different plant species under elevated CO_2 (Osborne et al. 1997; Lake and Hughes 1999).

In this study we therefore investigated effects of elevated CO_2 and nutrient availability on nectar production in *Epilobium angustifolium*, a classical study species in pollination biology since Sprengel (1793). However, regarding nectar production of *E. angustifolium*, we only found one study, which showed that sugar concentration increased linearly with decreasing air humidity, but that the rate of sugar production remained constant for the different humidity levels tested (Bertsch 1983). As the response of plants to elevated CO_2 may depend largely on nutrient availability (Curtis 1996; Poorter et al. 1996; Stöcklin and Körner 1999), we grew the test plants under two different fertilization regimes. Boose (1997) has shown genetically based variation in nectar production rates and significant genotype × environment interactions in *Epilobium canum*, a related, humming-bird pollinated plant species from California. However, to date, no study has investigated effects of elevated CO_2 on nectar production in different genotypes of a plant species. To address this aspect was a second aim of our study. The present study was carried out as a sister experiment to an investigation of effects elevated CO_2 and nutrient availability on pollen germination (Lavigne et al. 1999).

Materials and methods

The plant species

Epilobium angustifolium L. (Onagraceae) is a perennial, temperate clonal herb that typically colonizes nutrient-rich, open habitats. The plant perennates with roots. Shoots are produced annually and die back every year. Numerous, large protandrous hermaphroditic flowers are produced sequentially from the bottom to the top in an elongated raceme. Flowers are pollinated by insects, and outbreeding is strongly promoted by a marked protandry and because few flowers open simultaneously on an individual shoot. Selfing or geitonogamy is possible, however, but reduces seed production by ca 80% (J. Stöcklin; unpublished data). Outcrossed fruits contain up to 600 seeds.

Plants from five seed families were used in the experiment to investigate the influence of CO_2 concentration and two nutrient levels on nectar production and flower phenology, and to test for the existence of family × treatment interactions. Each of these families shared the same mother plant and differed only by the father plant used as pollen donor for hand pollinations. Seed families 1–4 were outbred, while family 5 resulted from the self-fertilization of the mother plant. Variation among families can be interpreted as nuclear-based genetic differences.

Experimental conditions

All plants were grown from seeds 2 years before the beginning of the experiment. Five 3-week-old seedlings (one per family) were placed in each of 24 26.5 × 21.5 cm containers filled with approximately 12 l of loamy soil (depth 20 cm) from an unfertilized meadow where *E. angustifolium* naturally occurs. Six of these containers were subjected to each of the four treatment conditions described below from April 1995 to July 1996 (two growing seasons), except during winter (November–February), when the containers were left in the open for vernalization.

The two CO_2 concentrations were maintained at 350 (current) and 650 (elevated) ppm in four (two each) naturally lit environmental chambers. Chambers were 17 m^3 polyethylene-covered chambers with a ground area of 6.7 m^2 built within a greenhouse. Plants were grown under controlled environmental conditions at 24°C 16 h light/16°C 8 h darkness, and the reduction of solar radiation in the green house was compensated by additional light from 1,000-W daylight halogen lamps. These were activated automatically when ambient photon flux density dropped below 180 μmol m^{-2} s^{-1} during the natural photoperiod. At canopy level, photon flux densities ranged from 600 to 1,100 μmol m^{-2} s^{-1} at midday on sunny days.

The high level of nutrients was provided by adding 25 ml of 1 N Hoagland's solution weekly to the irrigation water for 10 weeks during both years. The low nutrient treatment consisted of 25 ml of 0.5 N Hoagland's solution. The high and low treatments were the equivalent of the addition of, respectively, 50 and 25 kg ha^{-1} yr^{-1}.

Six containers each (three with low and three with high nutrient levels) were placed in the two current CO_2 chambers and six each in the two elevated CO_2 chambers. To avoid possible effects from heterogeneity within chambers or differences between chambers with different CO_2 levels, containers with plants were kept on rolling tables that were rotated weekly within the chambers. In addition, on the same day, containers were randomized on the tables. Finally, containers were moved every 2 months between chambers with the same level of CO_2.

Data collection

Data for this study were collected in the second year of CO_2 exposure when 112 of the 120 plants were flowering. Phenological observations were made from the onset of flowering and included the date of the first open flower of flowering plants and the course of anthesis of selected flowers used for nectar sampling. To standardize nectar sampling, samples from different plants were taken at the same time of day (8.00–10.00) and only if at least five flowers of the same age per individual plant were present. Sampling was performed during five consecutive days between 27 May and the 15 June. During this period samples were taken from 64 plants with sufficient flowers. From each plant, nectar samples were taken from five selected flowers per plant with carefully drawn out glass micropipettes flamed at the tip to avoid scratching the floral tissue. Samples were spotted onto a filter paper (Whatman no. 1). Nectar volumes were calculated as means per plant from the spot area of the five nectar samples on the filter paper (Baker 1979; Dafni 1992; Kerns and Inouye 1993). Sugar concentration and composition of three samples per plant in bloom were determined with the aid of high-performance anion exchange chromatography with pulsed amperometric detection (Martens and Frankenberger 1990). Calculations of the concentrations of individual nectar sugars were based on internal standardization with trehalose and were multiplied by nectar volumes to calculate total sugar per flower.

Amino acid concentrations (two samples per plant) were measured with the histidine scale developed by Baker and Baker (1980, see also Dafni 1992 and Kerns and Inouye 1993), and total amino acids calculated by multiplying nectar volumes by corresponding concentration values.

Statistical analysis

Mixed model analyses of variance were performed using the statistical package JMP, version 3.1 (SAS Institute, Cary, NC, USA). Effects tested were CO_2, fertilizer level, containers nested within CO_2 and fertilizer levels, seed families and their interactions. Containers were treated as random effects. The main CO_2 effect, fertilizer level and the CO_2 × fertilizer interaction were tested using variation among containers as error term. Seed families and their interactions with CO_2 and nutrient level were tested against the residual error of individual plants. A priori contrasts were used for testing the effects of elevated CO_2 within nutrient level. To test for significant effects of elevated CO_2 within genotypes we used one-way analysis of variance.

Results

Under elevated CO_2 nectar production per day increased by 51% ($p < 0.01$), total sugar per flower by 41% ($p < 0.01$) and total amino acids per flower by 192% ($p < 0.01$, Table 1, Fig. 1). These responses at the flower level did not depend on fertilization. Note that the overall increase in total sugar per flower was caused by the overall increase in nectar production, not by an increase in nectar sugar concentration (mean ± SE: low CO_2 = 1.748 g/ml ± 0. 081, high CO_2 = 1.845 g/ml ± 0. 187, n.s.). In contrast, the strong increase in total nectar amino acids was caused by both, the total increase in nectar production and an increase in concentration (mean ± SE: low CO_2 = 0.46 μmol/m ± 0.10, high CO_2 = 0.76 μmol/ml ± 0.11, $P < 0.05$). All other parameters tested (i.e., nectar sugar concentration, proportion of glucose/fructose and proportion of sucrose/(glucose + fructose)) were not significantly affected by elevated CO_2 and/or fertilization.

Even though we did not find a significant seed family × CO_2 interaction in any measured parameter (Table 1), the increase in nectar production per day under elevated CO_2 varied considerably among seed families from +10% (n.s., seed family 1) to +104% ($p < 0.01$, seed family 5) with two of the five seed families investigated showing significant responses (Fig. 2).

Table 1 Degrees of freedom, *F* values and significance levels from ANOVAs with data of nectar production per day, sugar production per day, the proportion glucose/fructose, the proportion sucrose/(glucose + fructose) and total amino acids in flowers of *Epilobium angustifolium*. Effects tested include CO_2 level (350 vs 650 μl^{-1}), fertilization (low vs high) and seed families nested within container and all interactions (*F* values with $p > 0.1$ are considered as n.s.)

Source	DF	Nectar per day	Total sugar per flower	Proportion glucose/fructose	Proportion sucrose/(glucose + fructose)	Total amino acids per flower	
CO_2 level (CO_2)	1	8.3**	5.3*	3.6 $p = 0.07$	0.7 n.s.	16.4***	
Fertilization level (fert.)	1		1.1 n.s.	0.7 n.s.	1.5 n.s.	0.2 n.s.	0.1 n.s.
CO_2 × fert.	1	0.4 n.s.	0.9 n.s.	0.1 n.s.	0.1 n.s.	0.5 n.s.	
Container	19						
Seed families	4	1.0 n.s.	0.2 n.s.	1.4 n.s.	1.9 n.s.	0.8 n.s.	
CO_2 × seed families	4	1.6 n.s.	1.6 n.s.	1.0 n.s.	1.8 n.s.	1.6 n.s.	
Fert. × seed families	4	0.6 n.s.	0.5 n.s.	1.0 n.s.	0.9 n.s.	0.3 n.s.	
CO_2 × fert. × seed families	4	0.2 n.s.	0.5 n.s.	0.3 n.s.	1.8 n.s.	0.4 n.s.	

Levels of significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

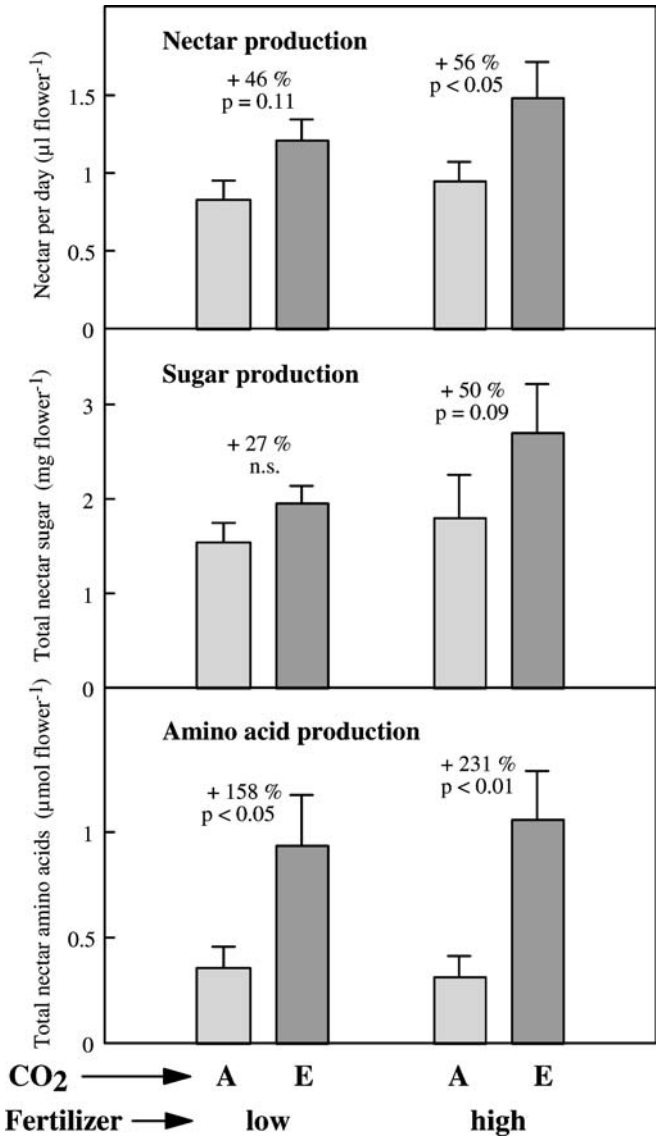

Fig. 1 Total nectar, nectar sugar and amino acid production per day (means ± SE) of flowers of *Epilobium angustifolium* for two CO_2 levels (current A, elevated E) and two fertilization levels (low vs high). Significant differences due to elevated CO_2 within fertilization treatments are indicated (*P* values from a priori contrasts)

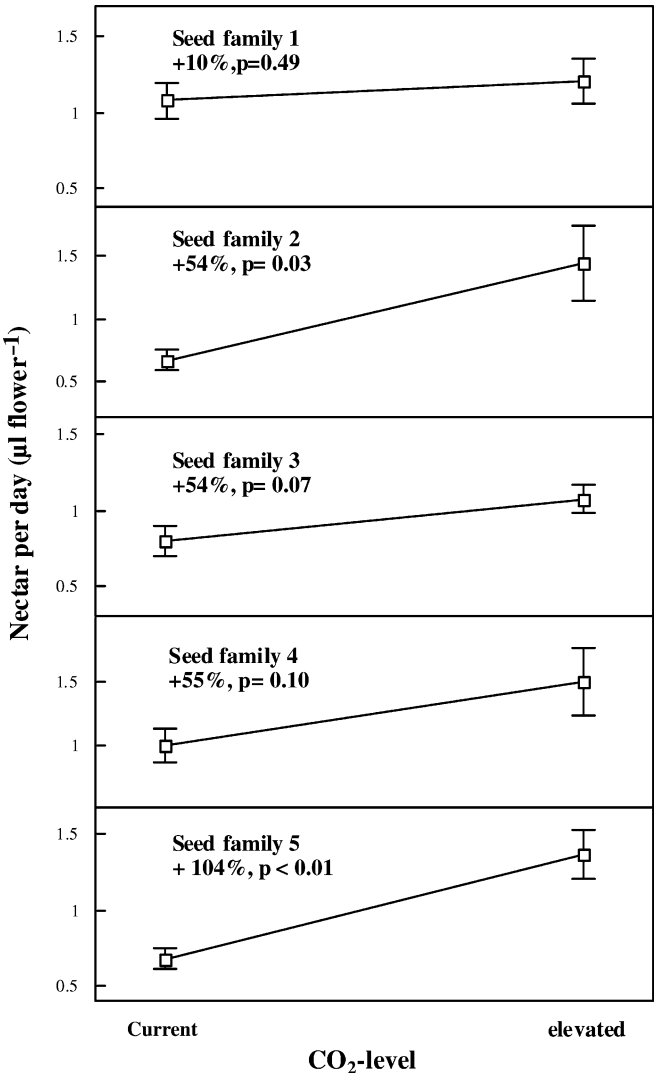

Fig. 2 Reaction norms of seed families of *Epilobium angustifolium* for current versus elevated CO_2 for flower nectar production per day (both fertilization levels pooled). Within each figure panel shows means ± SE for both CO_2 levels. Percentage response to elevated CO_2 and *P* values from one-way ANOVA for each seed family are indicated

The onset of flowering of *E. angustifolium* was not significantly affected by elevated CO_2 and/or fertilization. However, we found a marginally significant trend for earlier flowering at elevated CO_2 (2.4 days, $p < 0.06$, data not shown) in the highly fertilized plants, indicating a possible shift in the phenology of *E. angustifolium* under elevated CO_2 if sufficient nutrients are available.

Discussion

In contrast to other aspects of plant growth, effects of elevated CO_2 on floral parameters do not seem to have received adequate attention. In particular, investigations on nectar production and flowering phenology are rare. The present study shows that elevated CO_2 can positively affect nectar production rate, total nectar sugar, amino acid concentration and total nectar amino acids per flower. While a positive response in carbohydrate production under elevated CO_2 is expected, the corresponding increase in amino acid concentration and hence in total amino acids was contrary to the expected decrease of the N/C ratio under elevated CO_2. No clear explanation can be offered at present to this intriguing result. Interestingly, fertilization alone did not enhance amino acid concentration in *E. angustifolium*, in contrast to a corresponding study in *Agrostemma githago* (Gardener and Gillman 2001b). Even though the increase in total nectar amino acids is substantial, it must not be overinterpreted. *Epilobium angustifolium* is mainly pol-

linated by bees and bumblebees, which satisfy their needs for amino acids and nitrogen from collecting pollen, unlike, e.g., nectar-feeding butterflies, whose needs for amino acids in the adult stage must be fully satisfied by nectar. Thus, nectar of *E. angustifolium* has a rather low amino acid concentration in comparison to other plant species, and in spite of the increase in total amino acids under elevated CO_2, the level remains low. In fact, total amounts of nitrogen in nectar are small in comparison to the total nitrogen contents of a plant. We calculated a ratio of nitrogen in nectar to nitrogen per plant of less than $1{:}10^4$. Nevertheless, an increased offer of nectar and amino acids could be attractive to pollinating bees and bumblebees, and enhance frequencies of flower visits and hence outcrossing.

Variation in nectar production under elevated CO_2 in different genetic lines, although not further specified, has previously only been found in *Ipomoea purpurea* (Rathcke 1992). Though we only found positive responses, different seed families of *E. angustifolium* tended to react highly variably to elevated CO_2. However, we did not find a trade-off between nectar production and plant size or number of flowers produced under elevated CO_2 in the different seed families (data not shown). Together with results from other studies of components of fitness (Wulff and Alexander 1985; Ward and Strain 1997; Lavigne et al. 1999; Andalo et al. 2001), this confirms that there is selective potential under elevated CO_2 for particularly responsive genotypes of a species. For instance, Galen and Plowright (1985) reported that increased nectar rewards led to longer bumblebee tenure on flowers and greater pollen receipt in *E. angustifolium*, and that bees visited more flowers per plant on plants with more nectar. In other plant species higher nectar rewards also usually led to increases in components of plant fitness (e.g., Thomson 1986; Mitchell and Waser 1992; Mitchell 1993; Hodges 1995; Irwin and Brody 1999).

The fact that nectar quality was relatively little affected by elevated CO_2 and fertilization except for the moderate increase in amino acid concentration implies that nectar quality is a conservative floral trait in *E. angustifolium*. For instance, nectar sugar concentration may represent a result of selection by pollinators for concentrations, which maximize their feeding rate and energetic intake while foraging (see e.g., Kingsolver and Daniel 1979; Pyke and Waser 1981; Harder 1986; Roberts 1996), or a compromise between such selection and selection to minimize the energetically costly production of nectar (e.g., Pyke 1991).

In comparing *E. angustifolium* with previous studies, investigating effects of elevated CO_2 on nectar production, three features must be emphasized (Table 2). (1) Responses in nectar volume and total sugar per flower vary considerably among species, with some species responding positively (as expected) to elevated CO_2, but other species responding negatively, whereas yet another group of species remained indifferent. (2) In contrast to nectar volume and total sugar, sugar con-

Table 2 Overview of species examined for effects of elevated CO_2 on nectar production (0: no measurement, –: no effect)

	Ipomoea purpurea[a]	*Vicia faba*[b]	*Trifolium pratense*[c,d]	*Lotus corniculatus*[c,d]	*Betonica officinalis*[c,d]	*Scabiosa columbaria*[c,d]	*Centaurea jacea*[c,d]	*Tropaeolum majus*[e]	*Cucumis melo*[f]	*Epilobium angustifolium*
Nectar volume	increase	–	–	–	−28% (*)	−50%***	−38%**	−1 ± 250%***	+ ca 85%*	+ 51%**
Sugar concentration	–	–	–	–	–	–	–	–	–	–
Sugar composition	0	0	–	–	–	–	–	0	0	–
Total sugar/flower	0	0	–	–	–	−45%*	−31%*	–	+ ca 85%*	+ 41%**
Amino acid concentration	0	0	–	–	–	–	–	–	0	+ 65%*
Total amino acids/flower	0	0	–	–	−27% (*)	−40%***	−39%*	–	0	+ 192%**

[a] Rathcke 1992 (only qualitative results), [b] Osborne et al. 1997, [c] Erhardt and Rusterholz 1997, [d] Rusterholz and Erhardt 1998, [e] Lake and Hughes 1999, [f] Dag and Eisikowitch 2000.
(*) $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, [1] different subsamples.

centration and composition were not affected by elevated CO_2 in all species investigated so far. (3) Amino acid concentration and total nectar amino acids increased only in *E. angustifolium*. In all other species investigated, amino acid concentration was unaffected, and total amino acids decreased under elevated CO_2 in three of the seven investigated species. A recent attempt to correlate morphology and structure of nectaries with the response of different plant species to elevated CO_2 showed no clear relationship (Davis 2003).

Although we observed a trend for earlier onset of flowering in *E. angustifolium* under elevated CO_2, we have no mechanistic explanation for this trend at present, as plants did not reach their minimum size for flowering earlier under elevated CO_2.

Thus, we are far from drawing general conclusions about the effects of elevated CO_2 on floral phenology and nectar production. Interspecific differences in growth responses to elevated CO_2 are probably the rule and have been highlighted early (Bazzaz and Carlson 1984; Poorter 1993; Thomas and Jasienski 1996; Stöcklin and Körner 1999; Wieneke et al. 2004). Our results emphasize a trait of particular importance for plant–pollinator interactions. It appears that different plant species and probably also different genotypes within the same species react differently, with some species or genotypes increasing and some decreasing nectar quality and/or quantity under elevated CO_2. While our present findings suggest a potential fitness gain in *E. angustifolium* by increased nectar production under elevated CO_2, previous investigations on the other hand suggested potential shifts or even disruptions in fine-tuned plant–pollinator interactions. Further investigations may shed more light on this intriguing issue.

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