

## Breeding system of tristylous *Eichhornia azurea* (Pontederiaceae) in the southern Pantanal, Brazil

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**Abstract** The breeding system of *Eichhornia azurea* (Pontederiaceae) has been described as being both self- and heteromorphic incompatible based on crossing experiments performed on plants grown in an experimental garden. We studied the breeding system of tristylous *E. azurea* population under natural conditions in the Pantanal wetlands, Brazil. Controlled pollinations were conducted using 35 individuals of each floral morph. Legitimate pollinations produced more fruits than self- and illegitimate pollinations, except for the mid-styled morph which was highly self- and heteromorphic compatible. The number of seeds per fruit was higher under legitimate pollinations than in the other treatments, but self- and illegitimate pollinations produced more fruits and seeds in the Pantanal than for individuals of *E. azurea* in other populations. The higher fruit and seed production resulting from legitimate pollinations corroborate previous studies, but self-compatibility of mid-styled plants was not previously reported. Overall results indicate a partially self- and heteromorphic compatible system for this species in the Pantanal.

**Keywords** Aquatic macrophyte · *Eichhornia azurea* · Fruit production · Heterostyly · Seed set · Self-compatibility · Wetlands

### Introduction

Heterostyly is regarded as the most complex floral polymorphism because it provides both mechanical and genetic barriers to prevent autogamy (Vuilleumier 1967; Ganders 1979; Barrett and Husband 1990). Heterostylous species can exhibit either two (distyly) or three (tristyly) floral morphs that differ reciprocally according to the stigmas and anthers heights. Ancillary polymorphism of pollen and stigmas are generally associated with heterostyly and such differences are expected to be involved in self- and intra-morph incompatibility (Barrett and Cruzan 1994).

Tristyly is conspicuous in the Pontederiaceae, occurring in three of eight *Eichhornia* and four of five *Pontederia* species (Graham et al. 1998). However, heteromorphy and self-incompatibility appear to be stronger in the *Pontederia* than *Eichhornia* species. Most *Pontederia* species present evident pollen trimorphism, and populations are composed of three floral morphs occurring in equivalent proportions (Barrett 1977; Price and Barrett 1982; Barrett and Wolfe 1986, but see Barrett et al. 1983). However, the majority of tristylous *Eichhornia* species also have populations expressing a partial breakdown of heteromorphy and self-incompatibility resulting in monomorphic and dimorphic self-compatible populations and weak intermorph differentiation of pollen grains (Barrett 1978, 1979, 1985; Barrett and Harder 1992; Alves dos Santos 2002). The breeding system of *E. azurea* has been described to be heteromorphic self-incompatible in the Amazon, southern Brazil and northern Argentina (Barrett 1978; Alves dos Santos and Wittmann 2000; Bianchi et al. 2000). In addition, there are populations with semi-homostylous floral morphs (breakdown of tristyly) in Costa Rica and southeastern Brazil (Barrett 1978; Alves dos Santos 2002).

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The current paper presents information on the breeding system of *E. azurea* in the Pantanal, Brazil. All previous papers concerning the breeding system of *E. azurea* have been based on experimental data collected from plants removed from their natural environment. Barrett and Cruzan (1994) argued that temperature and humidity can affect pollen hydration and germination, and they suggested that results of breeding system experiments based on removed plants may not correspond to those in natural field conditions. Here we evaluate for the first time the occurrence of heteromorphic incompatibility in *E. azurea* under natural conditions, in the Pantanal wetlands, southwestern Brazil. We tested for differences among floral morphs in fruit and seed set from self-, illegitimate cross-, and legitimate cross-pollination treatments. We also addressed the question whether germination varies among seeds from each of the three floral morphs under different pollination treatments.

## Materials and methods

### Study site and species

The Pantanal is one of the largest wetland sites in the world (160,000 km<sup>2</sup>), included in the Ramsar Convention and was declared a World Biosphere Reserve and a Natural World Heritage Site by UNESCO in 2000. It comprises an extensive alluvial plain in the centre of South America. The climate is tropical with well-defined wet and dry seasons; mean annual rainfall is approximately 972 mm. It is an area with many ephemeral aquatic habitats, such as lagoons and oxbow lakes. Lagoons (a few centimetres to 2 m deep) are regionally called baías and are often densely covered by aquatic plants (Prance and Schaller 1982). In this study, we selected two permanent 1.5 m deep baías 4 km distant—hereafter referred as Baía 1 and Baía 2—each containing a high abundance of *E. azurea* inflorescences on the Rio Negro farm (19°34'S, 56°14'W), Aquidauana, Mato Grosso do Sul, Brazil. The proportion of short-, mid- and long-styled floral morphs was 2:1:2 ( $n = 1,000$ ) in Baía 1 and 1:1:1 ( $n = 129$ ) in Baía 2.

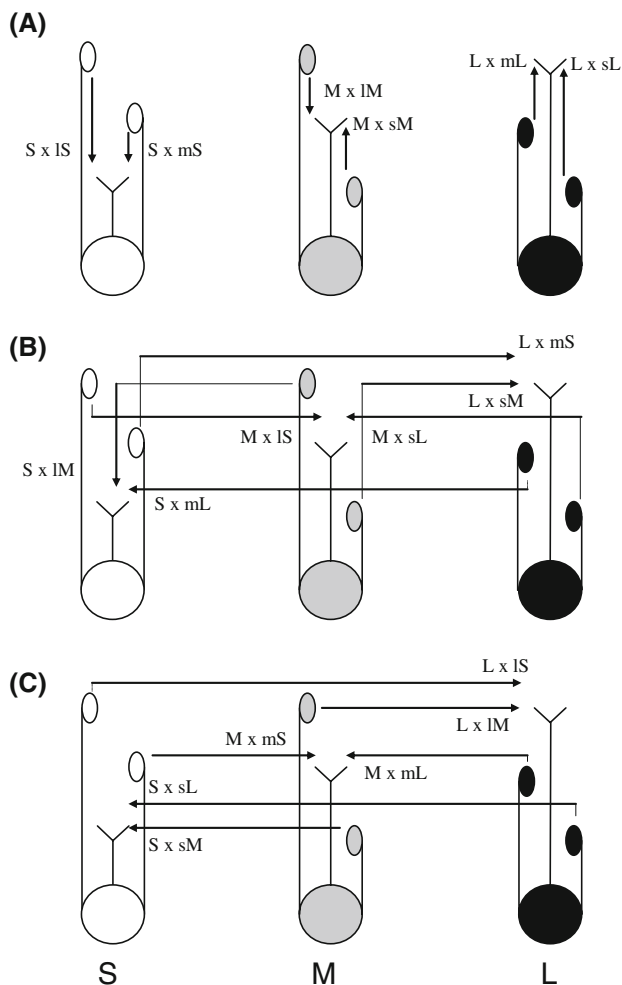
*Eichhornia azurea* is a perennial aquatic macrophyte with rhizomes, floating but fixed in the substrate (Pott and Pott 2000). It is found in aquatic environments of the Amazon, Paraguay, and Paraná basins in Brazil (Barrett 1978; Bianchi et al. 2000; Alves dos Santos 2002). Flowers are blue to purple and have six petals with fimbriate borders; nectar-guides are arranged in inflorescences. Three long and three short stamens are connected to the internal part of the floral tube. At the end of the flowering season, the inflorescence bends down and sinks into the water, where the fruits develop (Alves dos Santos 2002). Fruits are capsules containing hundreds of seeds (2 mm in length) (Pott and Pott 2000). Long-tongued solitary bees, flies, and

butterflies have previously been reported to be pollinators of *E. azurea* (Barrett 1978; Alves dos Santos and Wittmann 2000; Alves dos Santos 2002). In the southern Pantanal, opportunistic bees (*Apis mellifera*, *Trigona* sp.), butterflies, hemipterans and dipterans visit flowers of *E. azurea* (N. L. Cunha, personal observation).

### Data collection and analysis

For pollination treatments, we randomly chose plants in both baías situated at least 10 m apart with pre-anthesis buds, to avoid collection of individuals from potential clones. We selected 105 individuals: 18 plants of each floral morph in Baía 1 and 17 plants of each floral morph in Baía 2. Hand-pollination experiments were performed from November 2004 to February 2005. Plants used as pollen donors were chosen within 1 day of pollination experiments, before flower opening. When plants in Baía 1 were used as receptors of pollen, plants from Baía 2 were the pollen donors and vice versa, except for self-pollination treatments. This procedure avoids cross-pollination among potential clones in the same baía. All plants utilized in the pollination experiments were previously bagged to exclude floral visitors 1 day prior to the experiments. To prevent self-pollination, the long- and mid-anthers of the short floral morph and the long anthers of the middle floral morph were removed before hand pollinations. For each focal plant, six different hand-pollination treatments were performed: two self-pollinations, two illegitimate cross-pollinations and two legitimate cross-pollinations (Fig. 1a, b, c, respectively). We transferred pollen with forceps and a brush, which we cleaned after each experiment to prevent contamination among treatments. Immediately after hand pollinations the inflorescences were tagged and enclosed in fine-mesh bags to avoid insect visitation. Results of hand pollinations were taken 1 month later, when we collected infructescences in the field and brought them to the laboratory to count the number of fruits and seeds per fruit produced after each hand pollination treatment.

Five seeds of each fruit produced by the hand pollinations were randomly selected for germination tests. Seeds were placed in individual Petri dishes on filter paper with 5 ml of distilled water. The dishes were placed in a germination chamber set to an incubation temperature of 26.4°C and a photoperiod of 16/8 h. Seeds were inspected daily for germination for 15 days. Germination was considered successful if the radicle emerged. To test for differences in fruit production (dependent variable) among the three floral morphs and among pollination treatments (independent variables), a Pearson  $\chi^2$  test was performed. To test for differences in the number of seeds per fruit and the frequency of germinated seeds (dependent variables) among floral morphs and among pollination treatments



**Fig. 1** Hand-pollination treatments carried out among the three floral morphs (white, grey, and black) of *Eichhornia azurea* (Pontederiaceae) in the Pantanal wetlands; **a** self-pollinations, **b** illegitimate cross-pollinations, and **c** legitimate cross-pollinations. Anthers are represented by ellipses, ovaries by circles, and styles by Y-shaped lines. S, M, and L denote, respectively, short-, mid- and long-styled floral morphs. First letter in acronyms corresponds to the floral morph that is receiving pollen, and additional letters in the acronyms correspond to the pollen donor. For example, S × mL indicates that short-styled morph received pollen from mid anthers of the long-styled morph

(independent variables), we used a two-factor Analyses of Variance (ANOVA) and a posteriori Tukey tests for pairwise comparisons between means (Sokal and Rohlf 1995). Data on seeds per fruit were Poisson distributed, and therefore this variable was subjected to a square root transformation, and the percentage of germinated seeds were arcsine square root transformed (Sokal and Rohlf 1995).

## Results

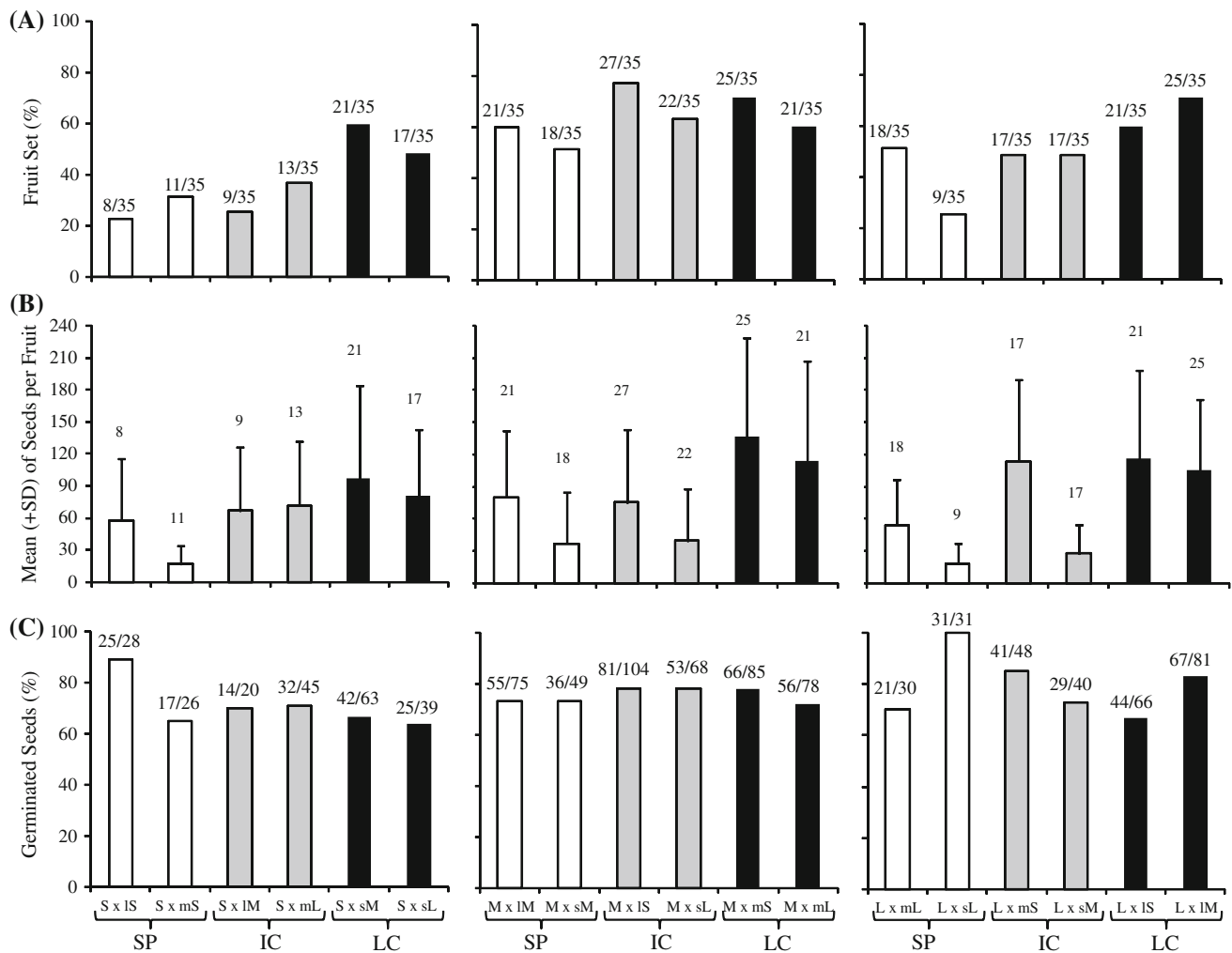
The success of fruit production among hand-pollinated flowers of *E. azurea* varied from 22 to 77% (Fig. 2a). The

proportion of fruits significantly differed among the three floral morphs (Pearson  $\chi^2 = 28.00$ ,  $df = 2$ ,  $P < 0.00001$ ). The short-styled flowers presented relatively low fruit production, the mid-styled ones had the highest fruit production, and the long-styled flowers showed intermediate values (Fig. 2a). The number of fruits differed among hand-pollination treatments for both short- and long-styled morphs. The legitimate cross-pollination treatments presented a greater number of formed fruits than treatments of illegitimate cross- and self-pollination. The long-styled flowers which received self-pollen from the short anther (L × sL), formed fewer fruits than all other treatments for this floral morph. Furthermore, self-pollinations (S × IS) and illegitimate cross-pollinations (S × IM) in this morph produced fewer fruits than the same treatments in the short-styled flowers. The observed proportion of fruits formed in the mid-styled flowers did not differ among the hand-pollination treatments (Fig. 2a).

The number of seeds per fruit did not differ ( $F_{2,319} = 1.235$ ,  $P = 0.29$ ) among the three floral morphs of *E. azurea*. However, seed set differed ( $F_{5,319} = 4.331$ ,  $P < 0.001$ ) among pollination treatments within each floral morph. The highest seed set occurred under legitimate cross-pollinations, followed by illegitimate cross-pollinations and by self-pollinations (Fig. 2b).

The number of seeds per fruit was influenced by the interaction of floral morph and pollination treatment ( $F_{10,319} = 6.869$ ,  $P < 0.0001$ ). In the S-morph, the legitimate cross-pollination with pollen from M-morph short anthers (S × sM) produced more seeds per fruit than the self-pollination with pollen from mid anthers (S × mS) (Tukey,  $P < 0.05$ ); and all other pollination treatments within this floral morph did not differ from each other. In the M-morph, seed production of the legitimate cross-pollinations via L-morph mid anthers (M × mL) and via S-morph mid anthers (M × mS) were higher than that of illegitimate cross-pollinations via L-morph short anthers (M × sL) and of self-pollinations via M-morph short anthers (M × sM) (Tukey,  $P < 0.05$ ). In the L-morph, legitimate cross-pollination via M-morph and S-morph anthers (L × IM and L × IS, respectively), produced more seeds per fruit than self-pollination via L-morph short anthers (L × sL) and illegitimate cross-pollinations via M-morph short anthers (L × sM) (Tukey,  $P < 0.01$ ).

Anthers of similar heights from different floral morphs did not show differing abilities to produce seeds for the two possible legitimate cross-pollination treatments (L × IM and L × IS, M × mL and M × mS, S × sL and S × sM; Tukey,  $P$  values varied from 0.96 to 1.00) nor for the two possible illegitimate ones (M × sL and L × sM, S × mL and L × mS, M × IS and S × IM; Tukey,  $P$  values varied from 0.98 to 1.00). Self-pollination treatments with pollen from lower-level anthers within each floral morph (short



**Fig. 2** Percentage of fruits produced per treatment (a), mean (+SD) of seeds per fruit (b), and percentage of germinated seeds (c) for the 18 hand-pollination treatments among the three floral morphs (short-, mid- and long-styled) of *Eichhornia azurea* (Pontederiaceae) in the Pantanal wetlands. Numbers above bars indicate number of fruits produced/number of hand-pollinated flowers (a), number of fruits in the samples (b), and number of germinated seeds/number of seeds

tested for germination (c). First letter in acronyms corresponds to the first letter of the floral morph that is receiving pollen, and additional letters in the acronyms correspond to the pollen donor. For example,  $S \times mL$  indicates that short-styled morph received pollen from mid anthers of the long floral morph. SP, IC, and LC indicate, respectively, self-pollinations (white bars), illegitimate cross-pollinations (grey bars) and legitimate cross-pollinations (black bars)

anthers in the M- and L-morph, and mid anthers in the S-morph) were less effective than the self-pollination using upper-level anthers (long anthers in the M and S-morph, and mid anthers in the L-morph); and than all legitimate and illegitimate pollination treatments.

The frequency of germinated seeds differed ( $F_{2,217} = 5.650$ ,  $P < 0.005$ ) among floral morphs (Fig. 2c). Seeds from L-styled plants had lower (Tukey,  $P < 0.05$ ) germination than seeds from S- and M-styled plants. In addition, there was higher variation in the frequency in germinated seeds among L-styled plants than among S- and M-styled plants. The frequency of germinated seeds did not differ ( $F_{5,217} = 0.595$ ,  $P = 0.70$ ) among hand-pollination treatments, nor was it influenced by the interaction of floral

morphs and pollination treatments ( $F_{10,217} = 0.355$ ,  $P = 0.96$ ).

## Discussion

The higher proportion of fruits produced via legitimate pollinations than via self- or illegitimate pollinations indicates that the breeding system of *E. azurea* tends to be self- and heteromorphic incompatible in the Pantanal. This view is also reinforced by evidence of a higher number of seeds in fruits originating from legitimate crosses than in fruits from other hand-pollination treatments. However, a partial breakdown of self-incompatibility seems to occur in

the Pantanal because considerable numbers of fruits and seeds were also produced via self- and illegitimate pollinations, and because the M-morph showed no differences for fruit and seed productions among hand-pollination treatments. The weak incompatibility in the studied *E. azurea* population might be pre-zygotic due to ancillary characters (Barrett 1988a, b; Scribailo and Barrett 1991). Differences in stigmatic papilla, pollen grains, and style tissue among floral morphs have been described for populations of *E. azurea* in the Amazon, and in South and Southeast Brazil (Barrett 1978; Alves dos Santos and Wittmann 2000).

The current study has shown weak self-incompatibility to exist in a trimorphic population of *E. azurea* in the Pantanal, contrasting with the strong self-incompatible breeding system of trimorphic populations of *E. azurea* in other regions (Barrett 1978; Alves dos Santos and Wittmann 2000; Bianchi et al. 2000). This difference prompts us to consider that trimorphic incompatibility of *E. azurea* varies among sites; or it varies depending upon whether pollination treatments are carried out in the field versus the laboratory (Barrett and Cruzan 1994). In *E. azurea* the short-styled morph appears to be more incompatible in self and illegitimate crosses than the other floral morphs, a pattern already found for *E. azurea* in the Paraná basin and for other tristylous Pontederiaceae, *E. paniculata*, *Pontederia cordata*, *P. rotundifolia* and *P. sagittata* (Ornduff 1966; Barrett 1977; Glover and Barrett 1983; Barrett and Anderson 1985; Manicacci and Barrett 1996; Bianchi et al. 2000).

The mid-styled *E. azurea* plants in the Pantanal can be considered self-compatible because they produced similar proportions of fruits among the three hand-pollination treatments, and more fruits and seeds per fruit than did the short- and long-styled flowers for illegitimate and self-pollination treatments. In addition there was no difference in fruit production between the anther levels used in self-pollinations within mid-styled flowers. These data indicate a relaxation of the heteromorphic self-incompatibility system that has not been previously reported in other studies of *E. azurea*. Although these studies reported a higher success of self-pollination in mid-styled flowers than in the other morphs, they still found mid-styled morph to be partially self-incompatible (Alves dos Santos and Wittmann 2000; Bianchi et al. 2000). Self-compatibility among mid-styled plants has also been found in some other tristylous species (Ornduff 1966, 1983, 1993; Glover and Barrett 1983; Barrett and Anderson 1985). It has been suggested that the weak or absent heteromorphic incompatibility among mid-styled flowers might be a pleiotropic effect of a supergene controlling the trimorphism (Charlesworth 1979; Barrett and Shore 2008).

In the Pantanal, *E. azurea* long-styled flowers were markedly self-incompatible when stigmas received pollen from the short anther; a similar result was found by Bianchi et al. (2000) studying long-styled clones of *E. azurea*. This result is possibly related to the amount of reserves stored in the small-sized pollen from short anthers, which may reduce its effectiveness when placed on long-styled flowers (Ganders 1979; Barrett and Anderson 1985; Scribailo and Barrett 1991). It is also supported by other results from our experiments. In self-pollinations pollen from short-level anthers was twice as successful when pollinating mid-styled flowers (fruits flower<sup>-1</sup> = 0.51, seeds fruit<sup>-1</sup> = 37.17) than long-styled flowers (fruits flower<sup>-1</sup> = 0.26, seeds fruit<sup>-1</sup> = 18.44). Likewise, illegitimate cross pollinations with short anthers pollinating mid-styled flowers produced a higher proportion of fruits per flower (0.63) and seeds per fruit (40.09) than when pollinating long-styled flowers (respectively, 0.49 and 27.24). In general, comparisons among anther heights throughout all hand-pollination treatments indicate that pollen from mid- and long anthers have greater success than pollen from the short anthers.

Long-styled *E. azurea* plants had a reduced frequency of germinated seeds, suggesting that this morph has a reduced fitness when compared to S- and M-morphs. This result contrasts with that for tristylous *Lythrum salicaria* (Lythraceae), which had an increased germination frequency in the L-morph than in the M- and S-morphs (Anderson and Ascher 2000). It indicates absence of a germination pattern guided by differential ability among floral morphs of heterostylous species. In addition, there were no differences in seed germination among pollination treatments, supporting the hypothesis that inbreeding does not affect germination ability of *E. azurea* in the Pantanal.

In summary, results from this study indicate that *E. azurea* is moderately self- and heteromorphic compatible in the Pantanal. This is in contrast to data reported for other trimorphic populations of this species in the Amazon, southern Brazil and northern Argentina (Barrett 1978; Alves dos Santos and Wittmann 2000; Bianchi et al. 2000). Self-compatibility for *E. azurea* has only previously been described for semi-homostylous populations that lack the specialized pollinators—long-tongued solitary bees, oligolectic on *E. azurea* (Alves dos Santos 2002). Annual flooding pulses may isolate and reconnect *E. azurea* populations in the Pantanal, and specialized pollinators of *E. azurea* seem to be absent there (N. L. Cunha, personal observation). Population isolation and ineffective pollinations may lead to the breakdown of the incompatibility system in heterostylous species (Vuilleumier 1967; Charlesworth 1979; Barrett 1988a, b; Husband and Barrett 1998). Further studies are needed to address these possible effects on the breeding system of *E. azurea* in the Pantanal.



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