SHORT COMMUNICATION

Fluctuating selection by water level on gynoecium colour polymorphism in an aquatic plant

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• *Background and Aims* It has been proposed that variation in pollinator preferences or a fluctuating environment can act to maintain flower colour polymorphism. These two hypotheses were tested in an aquatic monocot *Butomus umbellatus* (Butomaceae) with a pink or white gynoecium in the field population.
• *Methods* Pollinator visitation was compared in experimental arrays of equivalent flowering cymes from both colour morphs. Seed set was compared between inter- and intramorph pollination under different water levels to test the effect of fluctuating environment on seed fertility.
• *Key Results* Overall, the major pollinator groups did not discriminate between colour morphs. Compared with the white morph, seed production in the pink morph under intermorph, intramorph and open pollination treatments was significantly higher when the water level was low but not when it was high. Precipitation in July was correlated with yearly seed production in the pink morph but not in the white morph.
• *Conclusions* The results indicated that the two colour morphs differed in their tolerance to water level. Our study on this aquatic plant provides additional evidence to support the hypothesis that flower colour polymorphism can be preserved by environmental heterogeneity.

Key words: *Butomus umbellatus*, colour polymorphism, environmental heterogeneity, pleiotropic effects, pollinator preference, water stress.

INTRODUCTION

Pollinators are usually attracted to flowers initially by floral colour or scent, although most flowers reward pollinators with nectar or pollen as food (Proctor *et al.*, 1996). Pollinators may use these signals as cues of quality or quantity of the reward (Weiss, 1991; Meléndez-Ackerman *et al.*, 1997; Armbruster *et al.*, 2005, and references therein). Therefore, variation in flower colour may affect pollinator visitation rates and, in turn, plant reproductive success (e.g. Mogford, 1974; Waser and Price, 1981; Stanton, 1987; Rausher and Fry, 1993; Comba *et al.*, 2000; Gigord *et al.*, 2001; Jones and Reithel, 2001). For example, an experimental study in *Delphinium nelsonii* indicated that a reduction of seed set in white-flowered plants compared with pigmented plants was caused by pollinator discrimination (Waser and Price, 1981). Other studies have not observed pollinator discrimination against certain petal colours (Mogford, 1978; Hannan 1981; Miller 1981; Levin and Brack, 1995; Jones, 1996; Jersáková *et al.*, 2006). However, flower colour polymorphism could be maintained by fluctuating selection from variation in pollinator species (Brown and Clegg, 1984; Meléndez-Ackerman *et al.*, 1997; Subramaniam and Rausher, 2000; Turelli *et al.*, 2001; Eckhart *et al.*, 2006; Streisfeld and Kohn, 2007). A reciprocal transplant experiment of coastal red- and inland yellow-flowered races in *Mimulus aurantiacus* showed that hummingbirds preferred red flowers (>95 % of visits) but hawkmoths preferred yellow flowers (>99 % of visits) (Streisfeld and Kohn, 2007). Strong preferences for alternative floral morphs in

M. aurantiacus suggest that pollinators play a direct role in colour divergence.

On the other hand, flower colour polymorphism could be caused by pleiotropic effects or indirect selection on plant performance in contrasting physical environments (Mølgaard, 1989; Schemske and Bierzychudek, 2001, 2007; Chittka *et al.*, 2001; Warren and Mackenzie, 2001; Conner, 2002; Coberly and Rausher, 2003, 2008; Jorgensen and Andersson, 2005; Lacey and Herr, 2005; Strauss and Whittall, 2006). Studies have shown that floral pigments are associated with tolerance to environmental stress. For example, Warren and Mackenzie (2001) compared plant fitness in five flower colour polymorphic species under dry and well-watered conditions. They revealed that the anthocyanin-pigmented plants performed relatively better in the dry conditions, while the unpigmented (white-flowered) plants performed relatively better in the watered treatment (e.g. producing more seeds). A long-term study of field populations in *Linanthus parryae* found that blue-flowered morphs performed better than white-flowered morphs in drought years while white morphs performed better in years of high rainfall (Schemske and Bierzychudek, 2001, 2007).

Gynoecium colour polymorphism was recently discovered in an emergent, aquatic monocot *Butomus umbellatus* (Butomaceae). The species, native to Europe and north temperate Asia, usually has pink gynoecia, but plants with white gynoecia were observed in some populations in northeastern China (Huang and Tang, 2008). This gynoecium colour polymorphism provides a unique opportunity to understand the maintenance of colour polymorphism within populations,

given that colour variation in sexual organs may directly relate to plant reproductive success. Here two possibilities are addressed. (*a*) To examine the pollinator preference hypothesis we ask whether pollinators discriminate against an alternative colour morph. (*b*) To examine pleiotropic effects of the gynoecium colour polymorphism we ask whether plant performance differs under fluctuating environments. Seed production in *B. umbellatus* was found to vary with water level (Hroudova and Zakravsky, 2003). Thus, we address in particular whether the two colour morphs differ in tolerance to water stress as documented in previous studies (Schemske and Bierzychudek, 2001, 2007; Warren and Mackenzie, 2001).

MATERIALS AND METHODS

Study species and sites

Butomus umbellatus L. (Butomaceae) is an emergent aquatic herb usually growing in ponds, shallow water around the margins of lakes and slow-moving rivers. This species can reproduce both sexually through seed and clonally via rhizome fragmentation and vegetative bulbils, which are borne on rhizomes and occasionally on inflorescences (Eckert *et al.*, 2000). Plants flower in July to August and are pollinated by diverse insects. One plant can produce 1–4 cymose umbels on the upright approx. 1 m high stalks, and each umbel usually has 20–30 hermaphrodite flowers (Huang and Tang, 2008). Flowers consist of three pink sepals, three slightly larger pink petals, nine stamens and six conduplicate carpels, with nectaries at the base of the carpels. One flower generally lasts 3 or 4 d. It is strongly pro-tandrous with an intervening 1 d neuter phase during which no pollen is available and stigmas are not yet exposed (Bhardwaj and Eckert, 2001), permitting hand-pollination of flowers without emasculation. Seeds generally mature in 2–3 weeks after fertilization. This study focused on a population at Hexiangyuan wetland in Mishan City (N45°30′32″ E131°51′18″), in the northeast of China, in which there were >1000 individuals of both pink and white gynoecium morphs. Gynoecia in all flowers of one plant are either pink, as observed in other areas, or white, suggesting that gynoecium colour in this species is heritable rather than due to developmental plasticity or colour changes during growth. There was no significant difference in plant height, flower size or flower number per plant between the two morphs, but plants with pink gynoecia had wider inflorescence stalks, and more pollen and ovules than those with white gynoecia. The morph ratio was approx. to 1:1 in the studied population where a plot had 1144 pink and 1133 white individuals (Huang and Tang, 2008).

Pollinator preference

To test if pollinators prefer one morph to the other, pollinator visits to experimental arrays were observed for 2 years. The experimental array was established in two plots (1×1 m^2) with a 5 m interval between them from 11 to 14 July 2007 with an average 40 cm water level above mud. Two pink and two white individuals in one plot were set alternately into the four corners of these squares. Numerous flowers within umbels flower synchronously (Bhardwaj and Eckert, 2001). To reduce the effect of variation in floral display size, in each morph six male-phase flowers were retained in one umbel with removal of earlier flowers. Pollinator visits to the array (eight plants with 48 flowers) were recorded for an hour at a time and were observed for a total of 33 h over four fine days.

In July 2008, the population experienced heavy rain during flowering, and this permitted comparison of pollinator visitation in different weather conditions and with fluctuating water levels. Therefore, pollinator visits to arrays were observed at different water levels. For each observation, two hexagonal plots were set up, with a 5 m interval, in which each edge was 1·0 m long. Then six individuals were set to each plot, three pink and three white umbels alternately, in the six angles of each plot. Any two neighbouring individuals were different colour morphs. As in the array in 2007, each umbel had six male-phase flowers. Insect visits to one array (12 plants with 72 flowers) were observed from July 15 to 17 for a total period of 23 h when the water level was low (average 40 cm) and from July 23 to 26 for >20 h in the other plot when the water level was high (average 90 cm).

Effect of water level on morph performance

Water level has been considered as one key factor influencing yearly variation in seed production of *B. umbellatus* (Hroudova and Zakravsky, 2003). To examine whether seed production in the two morphs was affected by the fluctuating water level, we counted seeds per capsule from the field population for five consecutive years from 2005 to 2009 and noted the water level of the habitat. Each year we randomly collected one capsule each from at least 20 individuals per morph, except in 2009 when no flowers were produced by the pink morph. Monthly rainfall data in Mishan during the flowering season were kindly provided by the Weather Bureau at Mishan.

To compare the effect of water level on fertility between the two morphs, hand cross-pollination was conducted within and between two morphs at both a low water level (40 cm) and a high water level (90 cm) since the flower stalk of *B. umbellatus* can reach 100 cm (Huang and Tang, 2008). Before pollination treatments, one umbel of each of 15–20 plants of each morph was bagged using fine small-mesh (1×1 mm) nets to exclude insect visitation. In each umbel two flowers were pollinated: one received enough pollen from a pink morph and the other received pollen from a white morph. Pollen donors were randomly chosen from nearby plants of the appropriate morph. Late in the flowering season, we counted seeds per capsule from treated flowers as well as about 20 capsules randomly selected from open-pollinated umbels from the treated plants as a control.

Statistical analysis

To compare pollinator preference to the two colour morphs, *G*-tests were performed for goodness-of-fit of the null hypothesis that the relative visitation frequency of the two morphs is 1:1 in each of the arrays (random visitation). Seeds per fruit were normally distributed and heterogeneity was not observed. Therefore, a three-way analysis of variance (ANOVA) was

used followed by Tukey–Kramer's tests to analyse the effects of pollination treatments, gynoecium colour and water level on seed production. A two-way ANOVA was performed to test variation in seed production of open-pollinated flowers with year and morph as factors. Pearson correlation was performed to test the association between seed set and rainfall. Statistical procedures followed Sokal and Rohlf (1995). All the data were analyzed using SPSS 16·0.

RESULTS

Pollinator preference

A total of 5964 visits by the three major pollinator groups, bees, non-syrphid flies and syrphid flies, were recorded, accounting for 99 % of the total floral visits to the experimental arrays in 2 years. Spiders were occasionally observed on flowers but they did not act as pollinators. Flies were always the most frequent floral visitors in both high and low water level conditions, while bees only accounted for 5·9 % of the total floral visits (Table 1). Taken together, pollinator visits by the three groups in each array exhibited no discrimination between the two morphs ($G = 2·38$ for 2007 and 0·55 for 2008 at the low water level and 0·41 for 2008 at the high water level arrays, respectively; d.f. = 1, all $P > 0·1$). The relative frequency of each pollinator group was not significantly different from 1:1 expectations in the three experimental

TABLE 1. *Frequency of pollinator visits to pink and white morphs of* B. umbellatus *in experimental arrays*

Arrays	Pollinators	Visits to pink morphs	Visits to white morphs	G-test
2007	Bees	100	75	1·747
	Non-syrphid flies	695	520	3·480
	Syrphid flies	531	357	1·801
2008 low water	Bees	90	28	**27·955***
	Non-syrphid flies	692	670	0·004
	Syrphid flies	906	728	0·962
2008 high water	Bees	25	34	2·023
	Non-syrphid flies	100	80	1·042
	Syrphid flies	182	151	0·706

  G-tests report a test of the differences in visitation rates from 1:1 expectations.
  *$P < 0·05$.

arrays, except that bees preferred pink over white morphs in an array in 2008 but not in the other arrays (Table 1).

Fertility under different water level

Water level, gynoecium colour and pollination treatments significantly affected seed production in *B. umbellatus* (Table 2). The three-way ANOVA shows that there are significant interactions between colour morph and water level and pollination treatments, indicating differential roles of the water level and pollination treatments in the two morphs. Overall, seed production was significantly higher in the pink morph than in the white morph at the low water level ($F_{1,160} = 175. 928$, $P < 0·0001$) but not at the high water level ($F_{1,116} = 3. 592$, $P = 0·06$). Tukey–Kramer's tests indicate that intermorph pollination yielded more seeds than intramorph and natural pollination in both morphs. Within the pink morph, crossing with the white morph (intermorph pollination) produced more seeds than crossing with the pink morph (intramorph pollination) and natural pollination ($F_{1,282} = 57·744$; $P < 0·0001$), but there was no significant difference in seed number between intramorph pollination and open pollination (Fig. 1). Within the white morph, however, seed production was only marginally different between intermorph and intramorph pollination at a high water level ($F_{2,58} = 5·983$; $P = 0·043$), and was not significantly different among the three pollination treatments at a low water level ($F_{2,76} = 0·17$; $P = 0·844$).

Seed production of open-pollinated flowers varied among years (Table 3). In 2007 the yield of seeds was significantly greater and in 2009 the yield of seeds was significantly less than in the other years. These differences were largely attributed to the pink morph which produced the highest number of seeds in 2007 but the lowest in 2009. In July 2009 the population experienced almost 20 d of rain and the water level was on average >90 cm, and the pink morphs did not produce flowers at all. While seed production of the pink morph varied annually, the seed production of the white morph was relatively consistent (Fig. 2). Tukey–Kramer's tests indicate that yearly seed production in the white morph was only significantly different between 2006 and 2008 (high water level), but not between other years. In particular, seed production significantly decreased in the pink morph, when the water level was high in 2008 and 2009, suggesting that the pink morph was susceptible to fluctuating water levels.

Using the data for monthly precipitation in July from 2005 to 2009 in Mishan where *B. umbellatus* was located, a

TABLE 2. *Three-way ANOVA of the effects of water level, gynoecium colour and pollination treatments on seed production*

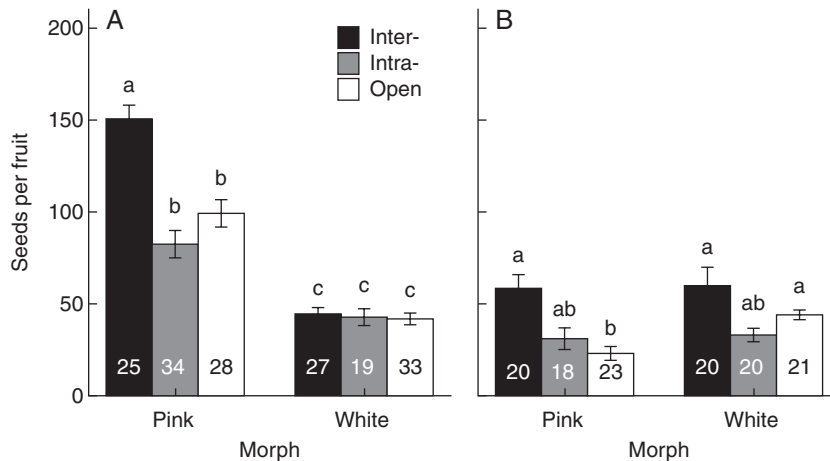| Variable	d.f.	MS	F	P
Water level	1	77 422·6	88·62	<0·0001
Colour	1	59 523·2	68·13	<0·0001
Pollination treatment	2	25 719·8	29·44	<0·0001
Water level × colour	1	100 387	114·90	<0·0001
Water level × pollination treatment	2	381·94	0·44	0·646
Colour × pollination treatment	2	8789·23	10·06	<0·0001
Water level × colour × pollination treatment	2	6191·25	7·09	0·001
Error	276	873·67		

FIG. 1. Comparison between two morphs of seeds per capsule (mean ± s.e.; number of replicates indicated on graph) under different pollination treatments at different water levels. The water level was on average 40 cm in (A) and 90 cm in (B). Different letters above the bars indicate significant differences in the two morphs.

TABLE 3. *Two-way ANOVA of 5-year data of seed production in the two morphs*

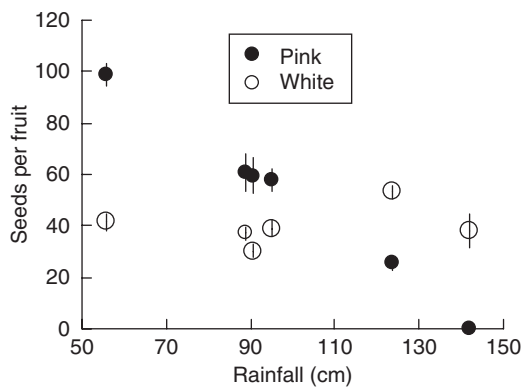| Variable	d.f.	MS	F	P
Year	5	10 984·3	22·28	<0·0001
Morph	1	6271·6	12·72	0·0004
Year × morph	5	16 367·6	33·20	<0·0001
Error	297	493·0		

FIG. 2. Seed production (mean ± s.e.) and rainfall in July in the two morphs over 5 years. Seed production in 2008 (rainfall approx. 90 cm) is shown at a low water level and a high water level.

significant negative correlation was found between rainfall and average seed production in the pink morph (Pearson correlation, $r = -0.998$, $P < 0.001$) but not in the white morph ($r = 0.269$, $P = 0.607$).

DISCUSSION

Our study demonstrated that all the major pollinator groups, flies and syrphid flies, did not discriminate between the two colour morphs in the three experimental arrays, although bees preferred pink over white morphs in one array. Seed production varied greatly with water level in the pink morphs but not in the white morphs. An investigation of reproduction in high water level conditions under artificial pollination showed that white morphs did not significantly decrease seed production whereas pink morphs did. The difference in tolerance to water stress between the two morphs suggests that the maintenance of a white gynoecium in *B. umbellatus* could be caused indirectly by pleiotropic effects rather than by pollinator-mediated selection.

Pollinators preferring one morph to the other may play a direct role in colour divergence. In some species it has been shown that strong pollinator preference for alternative floral morphs has resulted in biased female or male fertility (Waser and Price, 1981; Stanton, 1987; Streisfeld and Kohn, 2007). The open-shaped flower in *B. umbellatus* received generalist pollinators. It was found that visitation frequencies of the two major groups of pollinators, non-syrphid flies and syrphid flies which accounted for 94·1 % of the total floral visits, were not significantly different between the two morphs in the three arrays. We observed bees preferring pink morphs in one array, suggesting that gynoecium colour polymorphism is likely to be maintained by pollinator discrimination in this species if bees are effective pollinators. However, our observation of pollinator preference was based on small arrays that were only minimally replicated in this study. Further study is needed to quantify pollinator preference in large arrays and in various flowering seasons.

Compared with natural pollination, the artificial intermorph pollination increased seed production in the two morphs but intramorph pollination did not. The finding that intermorph pollination tended to yield more seeds than intramorph pollination (Fig. 1) may be due to biparental inbreeding within morphs, i.e. intramorph crosses may frequently have involved flowers of the same genotype in this species with extensive vegetative reproduction (Eckert *et al.*, 2003). Under artificial pollination treatments, each flower produced an average of 127 filled seeds in Ontario, Canada (Eckert *et al.*, 2000), and this amount of seed production is similar to our observation in the pink morph. Although seed set in three populations

could be increased from around 8 % to 20 % by supplemental pollination (Song, 1998), the cause of the generally low seed set (<20 %) in Eurasia remains unclear. Pollen grains deposited on the stigmas were more numerous than ovules in both pink and white morphs (X.-X. Tang, unpubl. res.), suggesting that pollinator abundance was not a key factor affecting seed production.

Pleiotropic effects have been considered to be associated with flower colour polymorphism. Studies showed that pigmented morphs had a better tolerance than the unpigmented (white) morph to harsh conditions such as drought (Warren and Mackenzie, 2001; Schemske and Bierzychudek, 2007), heat (Coberly and Rausher, 2003), plant vigour (Levin and Brack, 1995; Coberly and Rausher, 2008), herbivore or pathogen damage (Simms and Bucher, 1996; Irwin *et al.*, 2003) and edaphic stress (Rajakaruna and Bohm, 1999). Related species in the Hydrocharitaceae and Alismataceae are aquatic and most have white flowers, suggesting that pigmentation in *B. umbellatus* is a derived trait. Given that pigmentation involves a cost associated with anthocyanin production (Warren and Mackenzie, 2001), our finding that larger variation of seed production occurs in pigmented than unpigmented plants indicates that water stress acts as a potential factor affecting floral colour in this aquatic plant.

A fluctuating water level provides an opportunity to examine the response of two morphs to water stress. Compared with those flowering at a low water level, seed production in plants that experienced a heavy rainfall (>60 cm in a week, late July 2008) was significantly decreased in pigmented plants but not in white morphs. Such a decrease in seed production was not attributable to pollination failure in the rain because both artificial pollination and open pollination yielded lower seed production in the rain than at lower water levels. In the same population, which experienced heavy rain in early July 2009, pink morphs did not produce flowers at all. However, the white morphs produced flowers and yielded seed production that was approximately the same as that of other years (Fig. 2). The present results of seed production in the pink morph are consistent with an investigation by Hroudova and Zakravsky (2003), which indicated that seed production fluctuated from year to year depending on fluctuation of the water level. *Butomus umbellatus* generally grows in ponds where the water level was influenced by timely rainfall, causing fluctuating seed development in this species.

It was found that relative plant performance between pigmented and unpigmented individuals differed with the water level. The fertility of pink morphs was greatly influenced by the water level, with high seed production at a low water level. In contrast, individuals with a white gynoecium, although they usually produced fewer seeds, were less influenced in flooded conditions. The present result is mirrored in previous studies on terrestrial species (Schemske and Bierzychudek, 2001; Warren and Mackenzie, 2001), which showed that pigmented plants were more likely to grow well in a dry environment while unpigmented plants (white morphs) performed better in well-watered conditions. In conclusion, our study on an aquatic plant provides additional evidence in support of the hypothesis that flower colour polymorphism can be maintained by temporal or spatial heterogeneity of the environment.

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