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Effects of elevated temperature and CO₂ concentration on floral development and sex differentiation in *Morus alba* L.

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Abstract

• **Key message** Elevated temperature, elevated CO₂ concentration, and their combination significantly promoted the number and biomass of female mulberry (*Morus alba* L.) flowers, but the opposite is true for males. This paper demonstrates that male mulberry trees would suffer more negative effects on floral development and differentiation under global warming.

• **Context** With the ongoing intensification of global warming, flower formation has attracted widespread interest because it is particularly vulnerable to the effects of environmental factors. However, current knowledge of floral development regarding gender and sex differentiation under elevated temperature, CO₂ concentration, and their combination remains limited.

• **Aims** The aims of this study were to assess how sex-related differences in the morphology, biomass, and carbon (C) and nitrogen (N) contents of flowers respond to elevated temperature and CO₂ concentration.

• **Methods** *Morus alba* L. saplings (monoecious plants) were subjected to two temperature conditions (ambient vs. ambient + 2 °C) and two CO₂ regimes (ambient vs. ambient + 380 ppm CO₂) in growth chambers for 18 months growth, and differences in flowering phase, sex ratio, floral morphology and biomass, as well as total carbon and nitrogen of male and female inflorescences were investigated.

• **Results** Elevated temperature, elevated CO₂ concentration, and their combination significantly increased the number and biomass of female inflorescences but decreased the number and biomass of male inflorescences. Furthermore, the combination of elevated temperature and CO₂ concentration significantly decreased ovary length and C/N ratio of female flowers and the fresh weight of male flowers. Additionally, C/N ratio was negatively related to morphological traits of male inflorescences but positively related to tepal length of female flowers.

• **Conclusion** These findings indicate that global warming may affect floral development and sex differentiation in mulberry and that the male inflorescences of *M. alba* may suffer more negative effects than female inflorescences with respect to flower number, biomass, and morphological development.

Keywords C/N ratio; Floral morphology; Inflorescences; *Morus alba* L.; Sex ratio

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Contribution of the co-authors XX and TD designed the study and proposed the hypothesis tested; GH and GL analyzed the samples; DL and CZ analyzed the data and wrote the manuscript; all authors read and revised the final manuscript.

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1 Introduction

With the ongoing global warming, the effects of elevated temperature and atmospheric CO₂ concentration on floral development have increasingly drawn the attention of researchers. Changes in floral morphology (e.g., diameter, petals, pistils, stamens, and ovaries) and substantially reduced flower numbers were reported in response to increasing temperature in some crop and horticulture plant species (e.g., Rodrigo and Herrero 2002; Koti et al. 2004; Carvalho et al. 2005; Lucidos et al. 2013; Jagadish et al. 2016; Liang et al. 2017). Corolla, style, pedicel length, or inflorescence size increased with increasing temperature in *Vaccinium corymbosum* L., *Mangifera indica* L., and *Sandersonia aurantiaca* L. (Lyrene 1994; Sukhvibul et al. 1999; Catley et al. 2002), whereas pollen and flower number were unaffected by moderately elevated temperature in *Lycopersicon esculentum* Mill. and *Impatiens walleriana* Hook. f. (Sato et al. 2006; Vaid and Runkle 2013). In addition, the length of flower stem and the numbers of pigments, pollen, and flowers were markedly increased under higher CO₂ concentrations in *Ambrosia artemisiifolia* L., *Eustoma grandiflorum* (Raf.) Shinn., *Rosa hybrida* L., and *Viola × wittrockiana* Gams. (Niu et al. 2000; Ziska and Caulfield 2000; Ushio et al. 2014; Naing et al. 2016), whereas the number of flowers per flower head was not significantly affected by CO₂ concentration in *Lolium perenne* L. and *Trifolium repens* L. (Wagner et al. 2001). These results indicate that the morphology and the number of flowers display species-specific responses to enhanced temperature and elevated CO₂.

On the other hand, sex differentiation of flowers was also closely related to temperature, CO₂ concentration, and carbon to nitrogen ratio (C/N) (Korpelainen 1998; Wang et al. 2001; Chen et al. 2005; Hedhly et al. 2009). Higher temperature results in an increase in the number of male flowers (Sage et al. 2015), whereas elevated CO₂ results in the production of more female-biased progeny in *Silene latifolia* cv Poiret (Wang 2005); higher C/N ratio promotes male tendency while lower C/N ratio inhibits male flower development in bitter melon (*Momordica charantia* L.) due to its mediating role in the production of gibberellic acid, indole acetic acid, and dihydrozeatin which all have positive influence on flower formation (Kossuth and Ross 1987; Wang et al. 2001; Talamali et al. 2003; Glawe and Jong 2005). These studies indicate that sex differentiation in flowers is determined not only by genotype and phytohormones but also by C/N ratio and environmental factors (Yamasaki et al. 2000; Wang et al. 2001; Deputy et al. 2002; Khryanin 2002; Wu et al. 2010; Gerashchenkov and Rozhnova 2013; He et al. 2017).

Moreover, although the mechanism of sex differentiation in plants is still unclear, dioecious species evolved from the monoecious species through sexual specialization (Barrett 2002; Dorken and Barrett 2004; Ehlers and Bataillon 2007). Mulberry (*Morus alba* L.), an important tree in sericulture

and the silk industry in China, Japan, and India, is a highly heterozygous plant and shows sexual polymorphism (mainly dioecious or occasionally monoecious) (Tikader et al. 1995; Thomas 2004; Qin et al. 2018). To date, studies on mulberry have mainly focused on plant growth (Fukui 2000; Zeng et al. 2016), leaf quality (Chaitanya et al. 2001; Yu et al. 2013; Zeng et al. 2016; Sarkar et al. 2017), and physiological traits (Chaitanya et al. 2002; Ke et al. 2009), whereas there has been limited research on floral development and sex differentiation. Since flower formation is particularly vulnerable to the effects of environmental factors (Korpelainen 1998; Stehlik et al. 2008; Hedhly et al. 2009; Buide et al. 2018), we hypothesized that flower morphology and sex differentiation in those plant species with sexual polymorphism would be changed by global warming. To test our hypotheses, the sex-related differences in flower number, flowering phase, biomass, morphological traits, as well as the contents of carbon and nitrogen of mulberry flowers under elevated temperature, elevated CO₂, and their combination were investigated. The aim was to determine how sex-related differences in the morphology, biomass, and carbon and nitrogen contents of flowers could respond to elevated temperature and CO₂ concentration.

2 Materials and methods

2.1 Plant material and experimental design

On February 15, 2015, a total of 45 *M. alba* cuttings (10 cm in length) were collected from 15 mature trees (2 genotypes) growing in the germplasm base of the Sericultural Research Institute, Sichuan Academy of Agricultural Sciences (30° 52' N, 106° 04' E; 256 m above sea level). These trees are normally monoecious with separate male and female inflorescences on the same plant. Each of these cuttings was planted in a seedbed at the China West Normal University (30° 48' N, 106° 04' E; 276 m above sea level) in Nanchong, Sichuan Province, China. The annual mean rainfall, temperature, and insulation time in this area are 1065 mm, 16.8 °C, and 1980 h, respectively (Luo and Zhou 2007). After sprouting and growing for 5 months, 32 healthy saplings with similar size and height were selected and replanted in 10 L (30 cm × 24 cm) plastic pots (one sapling per pot) filled with 10 kg sandy soil (sand:soil = 1:1). The soil was a Cambisol (pH 8.0) obtained from the experimental site, which contained 10.9 g kg⁻¹ organic carbon, 0.76 g kg⁻¹ total nitrogen, 0.89 g kg⁻¹ total phosphorus, and 77.0 mg kg⁻¹ available potassium (Chen et al. 2016; Huan et al. 2016).

The experimental layout was completely randomized with two factors (temperature and CO₂ regime). Two temperature conditions (ambient vs. ambient + 2 °C) and two CO₂ regimes (ambient vs. ambient + 380 ppm CO₂) were applied. Four small growth chambers were used for the ambient temperature/CO₂ (control), elevated temperature, elevated CO₂ concentration, and

elevated temperature + elevated CO₂ concentration treatments. The chambers were approximately cylindrical structures with an internal volume of approximately 25 m³ and a ground area of 9 m². The chambers were constructed of glass walls with polycarbonate plastic tops and transmitted approximately 90% of the available light. The computer-controlled temperature and CO₂ system (SIEMENS TD400C V2.0; Yisheng Taihe Science and Technology Co. Ltd., Beijing, China) enabled automatic adjustment of temperature and CO₂ concentration within the chambers according to the ambient conditions. Eight saplings in each chamber and moving treatments among chambers every 30 days were used to minimize random errors. To ensure that the experimental plants received uniform illumination, their positions were rotated weekly. Moreover, all the pots were watered every 2 days with the same amount of water (about 500 mL) to maintain constant soil moisture (soil water content was always kept at 26.2%; about 92% field capacity). The treatment lasted 18 months (started on October 20, 2015 and ended on April 30, 2017).

2.2 Plant morphology

Four saplings were randomly selected from each treatment at the end of the experiment, and the shoot height and basal diameter of each sapling was then measured with a meter stick, respectively.

2.3 Flowering phase and sex ratio measurements

To document flowering phenology, four saplings were randomly selected and marked prior to flowering (started on February 1, 2017). The flowering status of each plant was recorded at 2-day intervals during the flowering phase. The initiation of flowering was defined as the day when the first flower opened, and the last day of flowering was defined as the day when the last flower wilted on the inflorescence. The flowering monitoring continued throughout the spring (ended on April 30, 2017). The flower sex ratio was calculated as the number of female inflorescences divided by the number of male inflorescences at whole plant level.

2.4 Floral morphology and biomass

During anthesis, four saplings were randomly selected from each treatment, and five male or female inflorescences (single-sex inflorescences) per sapling were randomly selected from the middle of the stem (according to Yang et al. 2014). The inflorescences were carefully cut from the stems in the morning (08:30–09:30). The length and fresh weight of each inflorescence were measured using a digital calliper (0.01 mm accuracy) and an electronic analytical balance with 0.0001 g accuracy (FA2004B; Shanghai, China), respectively. Prior to the removal of flowers from the inflorescence, the number of

flowers per inflorescence was counted. Three male or female flowers per inflorescence were randomly selected, weighed, and dissected under a stereoscopic microscope equipped with a charge-coupled device camera (SMZ-168-TL; Motic, Xiamen, China). The sizes of tepals, anthers, ovaries, and stigmas were measured to the nearest 0.01 mm using an ocular reticle. To measure the total dry mass of inflorescences per plant, all of the inflorescences on each selected sapling were cut from the stem at late blossom. The samples were oven-dried at 70 °C for 48 h to a constant weight and weighed.

2.5 Total carbon and nitrogen

At late blossom, male and female inflorescences from each treatment were collected and oven-dried at 70 °C for 48 h to a constant weight. The samples were ground in a mortar and passed through a 40-mesh screen. The carbon and nitrogen contents were quantified using a Vario MAX CN analyzer (Elementar Analysensysteme GmbH, Hanau, Germany).

2.6 Statistical analysis

Differences among means were analyzed using Duncan's multiple range test following one-way ANOVA at a significance level of $P < 0.05$. Two-way ANOVAs were used to evaluate the effects of temperature, CO₂ concentration, and their combination. An independent-sample *t* test was employed to determine differences between male and female inflorescences. Pearson's correlation coefficients were calculated to assess the relationships between each of the element variables (carbon, nitrogen, and the ratio of carbon to nitrogen) and the morphological traits of male or female flowers. All analyses were carried out using the SPSS 19.0 for Windows statistical software package (SPSS Inc., Chicago, IL, USA).

3 Results

3.1 Plant growth under elevated temperature and CO₂ concentration

Overall, compared with ambient conditions, the growth of *M. alba* saplings was significantly promoted by the combined use of elevated temperature and CO₂, and exhibited higher shoot height and basal diameter at the end of the experiment (Fig. 1; Appendix Fig. 7).

3.2 Flowering phase and sex ratio of flowers under elevated temperature and CO₂ concentration

The ratio of female to male flowers was significantly increased under elevated temperature, elevated CO₂ concentration, and the combined treatment (Fig. 2). A significant difference was observed between the expression

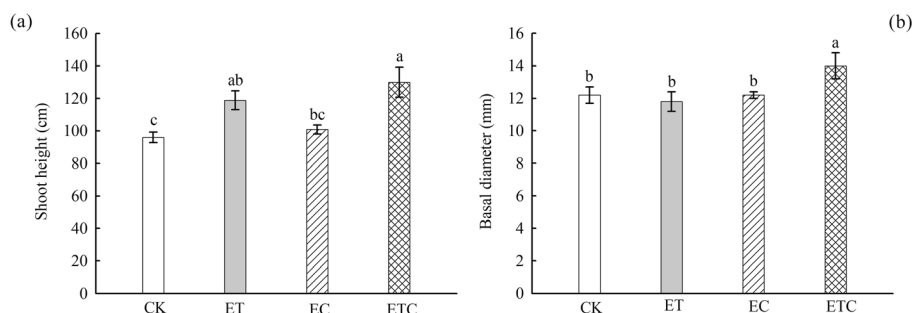


Fig. 1 The effects of elevated temperature, elevated CO₂ concentration, and their combination on shoot height and basal diameter in *Morus alba* L. after 18 months growth. CK control, ET ambient + 2 °C, EC ambient +

380 ppm, ETC combined ET/EC conditions. Different lowercase letters above bars indicate significant differences among treatments according to one-way ANOVA followed by Duncan's test ($P < 0.05$)

of male and female structures during the flowering phase, with male flowers (approximately 7 days) consistently showing a shorter developmental stage than female flowers (approximately 16 days) in each treatment. Furthermore, the flowering phase of male inflorescences was significantly increased in response to the elevated temperature and CO₂, whereas we detected no significant difference in the response of female inflorescences among the different treatments (Fig. 3).

3.3 Morphological traits of female and male flowers under elevated temperature and CO₂ concentration

At whole plant level, there were significant differences in the number and length of inflorescence and the number of flowers per inflorescence between male and female inflorescences (Table 1, Fig. 4). In controls, the number and length of male inflorescences were larger than those of female inflorescences. In contrast, under enhanced temperature, elevated CO₂, and

combined elevated temperature and CO₂, there were significantly higher numbers of female inflorescences and flowers per female inflorescence compared with male inflorescences. At the individual plant level, the number of female inflorescences was significantly increased by elevated temperature and CO₂ concentration ($P < 0.001$), whereas the number of male inflorescences was significantly decreased ($P < 0.05$; Table 1). Moreover, the length, flower number, and fresh weight of male inflorescences were significantly decreased by increased temperature, elevated CO₂ concentration, and combined ($P < 0.05$), whereas the corresponding traits in female inflorescences did not vary among treatments (Table 1). At flower level, only ovary length differed among treatments, showing a significant declining trend under elevated CO₂ concentration relative to the control ($P < 0.05$), whereas for male flowers, tepal length and fresh weight of single flowers were significantly decreased by increased temperature ($P < 0.05$; Table 2, Fig. 4).

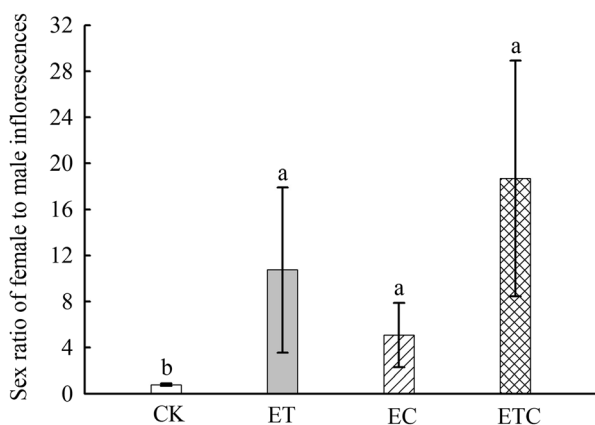


Fig. 2 The effects of elevated temperature, elevated CO₂ concentration, and their combination on sex ratio in *Morus alba* L. CK control, ET ambient + 2 °C, EC ambient + 380 ppm, ETC combined ET/EC conditions. Different lowercase letters above bars indicate significant differences among treatments according to one-way ANOVA followed by Duncan's test ($P < 0.05$)

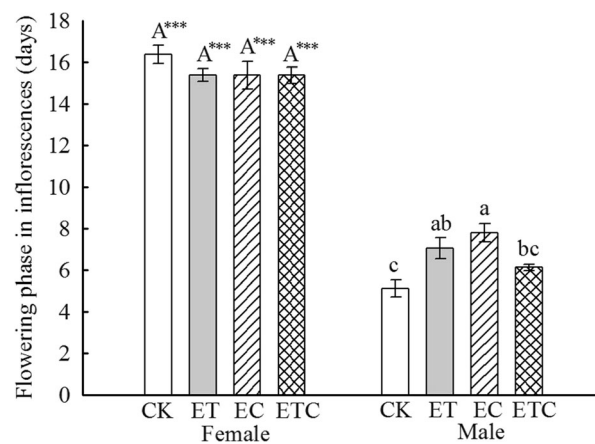


Fig. 3 The effects of elevated temperature, elevated CO₂ concentration, and their combination on flowering phase in *Morus alba* L. CK control, ET ambient + 2 °C, EC ambient + 380 ppm, ETC combined ET/EC conditions. Different letters above bars for the same sex group indicate significant differences among treatments according to one-way ANOVA followed by Duncan's test ($P < 0.05$). Asterisks above bars denote statistically significant differences between the sexes at $P < 0.05$ according to independent-samples t test ($***P \leq 0.001$)

Table 1 The effects of elevated CO₂ concentration, elevated temperature, and their combination on the morphological characters of inflorescences in *Morus alba* L.

Trait	Treatment					<i>P</i>		
		CK	ET	EC	ETC	<i>P_T</i>	<i>P_C</i>	<i>P_{T×C}</i>
Female	No. of inflorescences per plant	7.5 ± 3.6D *	116.8 ± 17.5B **	72.0 ± 15.2C ns	176.5 ± 14.8A ***	< 0.001	0.001	0.867
	Avg. length per inflorescence (mm)	14.9 ± 1.4A **	12.9 ± 1.5A ns	12.2 ± 1.4A ns	12.1 ± 0.8A ns	0.432	0.210	0.469
	No. of flowers per inflorescence	35.6 ± 4.6A ns	28.8 ± 3.6A *	30.8 ± 4.4A ns	31.4 ± 1.8A **	0.422	0.770	0.347
	Fresh inflorescence weight (mg)	97.2 ± 22.3A ns	69.7 ± 11.6A ns	59.4 ± 9.7A ns	63.2 ± 5.7A ns	0.407	0.133	0.279
Male	No. of inflorescences per plant	83.8 ± 15.8a	30.8 ± 12.6b	36.0 ± 16.4b	17.8 ± 5.7b	0.020	0.042	0.216
	Avg. length per inflorescence (mm)	22.1 ± 1.3a	13.2 ± 1.9b	14.6 ± 0.7b	13.9 ± 0.7b	0.003	0.019	0.008
	No. of flowers per inflorescence	32.3 ± 1.6a	17.2 ± 3.1b	21.8 ± 2.0b	17.1 ± 2.1b	0.001	0.037	0.039
	Fresh inflorescence weight (mg)	145.6 ± 6.8a	71.4 ± 17.3b	78.3 ± 8.0b	62.9 ± 4.3b	0.001	0.003	0.015

CK control, ET ambient + 2 °C, EC ambient + 380 ppm, ETC combined ET/EC conditions. Each value is the mean ± standard error. *P_T*, temperature effect; *P_C*, CO₂ effect; *P_{T×C}*, temperature × CO₂ effect. Different letters in the same row indicate significant differences among treatments according to one-way ANOVA followed by Duncan's test ($P < 0.05$; significant effects in bold). Asterisks following capital letters denote statistically significant differences between the sexes at $P < 0.05$ according to independent-samples *t* test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns not significant)

3.4 Biomass of female and male inflorescences under elevated temperature and CO₂ concentration

In the plants exposed to increased temperature, elevated CO₂, and their combination, female inflorescences displayed a higher total biomass than male inflorescences (Fig. 5). Moreover, the total inflorescence biomass per plant was significantly increased relative to the control in response to elevated temperature, whereas in plants exposed to the combined treatment, there was a significant increase in female inflorescence biomass but a significant decrease in that of male inflorescences ($P < 0.05$; Fig. 5).

3.5 Nitrogen contents and C/N ratios in female and male inflorescences under elevated temperature and CO₂ concentration

Under all treatments, nitrogen content of inflorescences was higher in males than in females (Fig. 6a), while the C/N ratio of inflorescences was lower (except combined treatments) in males than in females (Fig. 6b). Furthermore, elevated temperature, elevated CO₂, and combined treatments resulted in an increase in the nitrogen content of females, but a decrease in males (Fig. 6a).

3.6 Relationships among nutrient, mass, and morphological characters of female and male inflorescences

In female flowers, we detected a negative correlation between carbon content and inflorescence number, and nitrogen content is negatively correlated to tepal length (Table 3). In male flowers, single flower number and fresh weight were positively correlated with carbon and nitrogen contents, but negatively correlated with the C/N ratio (Table 3).

4 Discussion

Plant size is an indicator of resource statuses of a given stage (Shaanker and Ganeshaiah 1984). Although most plants need to reach mature age (or accumulate enough substances) before they can bloom, mulberry trees grown from cuttings have the capacity to bloom at the seedling stage (Okabe 1986). This flowering capacity (especially female flowers number) could increase with plant size, but it also could be triggered by environmental stresses (Clay 1993; Méndez 1998; Trusov and Botella 2006). In present study, *M. alba* saplings were used as experimental materials and exposed to higher temperature and CO₂ concentration treatment for 18 months growth, which make it possible to study the floral development and sex differentiation.

Our results are consistent with the findings of previous studies that higher temperatures favor predominantly female flowering in mulberry (*Morus* spp.) (Minamizawa 1963) and promoted an increase in the proportion of female flowers (Sage et al. 2015). Similarly, a warm temperature was favorable for female sex expression in *Silene littorea* Brot. (Buide et al. 2018). In contrast, however, other studies have reported that the development of male flowers in cucurbits and cucumber is promoted by high temperatures (Miao et al. 2010). This phenomenon is closely related to sex determination, which involves diverse mechanisms at the genic, genomic, epigenetic levels, hormones, as well as the specific life history traits and adaptation strategies of each species (Dellaporta and Calderon-Urrea 1993; Charlesworth 2002; Stehlik et al. 2008; Munné-Bosch 2015; Hobza et al. 2018). For example, the *SpGAI* expression of sex-determining genes and GA content is strongly modified by environmental factors (Retuerto et al. 2018; West and Golenberg 2018). Hence, our results provide evidence that global warming may lead to a predominance of female flowers and female bias in inflorescences in *M. alba* plants.

Fig. 4 The morphological and anatomical structures of male and female flowers of *Morus alba* L. (The scales beside the photographs is provided for scale only; the structures were measured with a micrometer)

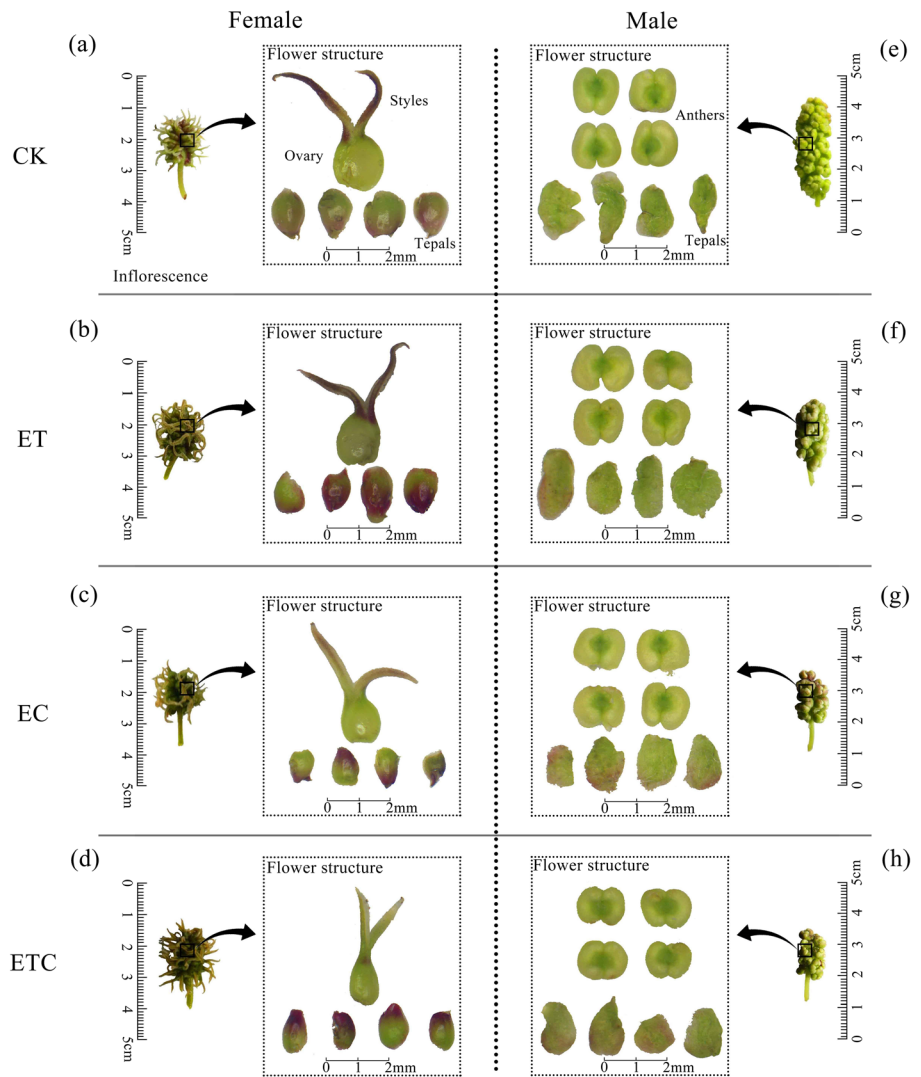


Table 2 The effects of elevated CO₂ concentration, elevated temperature, and their combination on the morphological characters of single flowers from *Morus alba* L.

Trait		Treatment				<i>P</i>		
		CK	ET	EC	ETC	<i>P_T</i>	<i>P_C</i>	<i>P_{T×C}</i>
Female	Tepal width (mm)	0.89 ± 0.06A	0.79 ± 0.05A	0.79 ± 0.04A	0.78 ± 0.04A	0.258	0.261	0.329
	Tepal length (mm)	1.56 ± 0.10A	1.29 ± 0.13AB	1.29 ± 0.04AB	1.26 ± 0.06B	0.116	0.119	0.206
	Ovary diameter (mm)	1.37 ± 0.10A	1.32 ± 0.14A	1.23 ± 0.03A	1.14 ± 0.07A	0.442	0.115	0.836
	Ovary length (mm)	1.63 ± 0.09A	1.61 ± 0.16AB	1.43 ± 0.01AB	1.34 ± 0.03B	0.470	0.018	0.689
	Fresh flower weight (mg)	2.09 ± 0.31A	2.03 ± 0.41A	1.67 ± 0.18A	1.53 ± 0.12A	0.714	0.120	0.885
Male	Tepal width (mm)	1.10 ± 0.06a	1.17 ± 0.02a	1.07 ± 0.04a	1.11 ± 0.06a	0.250	0.342	0.738
	Tepal length (mm)	1.61 ± 0.04ab	1.38 ± 0.05c	1.66 ± 0.08a	1.46 ± 0.06bc	0.003	0.291	0.765
	Anther width (mm)	1.41 ± 0.02a	1.54 ± 0.07a	1.45 ± 0.05a	1.38 ± 0.08a	0.616	0.339	0.105
	Anther length (mm)	1.25 ± 19.0a	1.21 ± 0.07a	1.23 ± 0.04a	1.14 ± 0.08a	0.411	0.447	0.827
	Fresh flower weight (mg)	5.24 ± 0.10a	4.61 ± 0.36b	4.61 ± 0.47b	4.30 ± 0.42b	0.002	0.075	0.068

CK control, ET ambient + 2 °C, EC ambient + 380 ppm, ETC combined ET/EC conditions. Different letters in the same row indicate significant differences among treatments according to one-way ANOVA followed by Duncan’s test (*P* < 0.05; significant effects in bold)

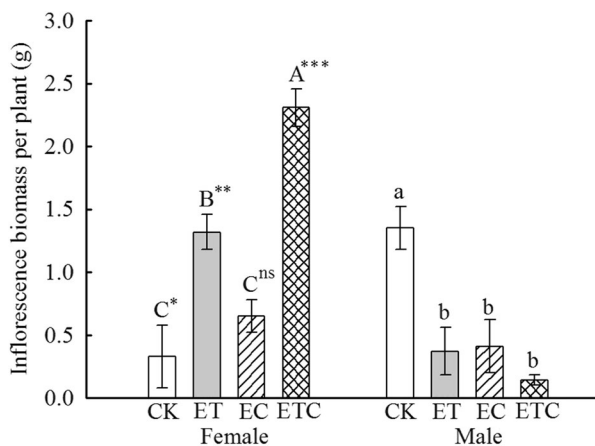


Fig. 5 The effects of elevated temperature, elevated CO₂ concentration, and their combination on biomass (dry mass) in *Morus alba* L. CK control, ET ambient +2 °C, EC ambient +380 ppm, ETC combined ET/EC conditions. Different letters above bars for the same sex group indicate significant differences among treatments according to one-way ANOVA followed by Duncan's test ($P < 0.05$). Asterisks above bars denote statistically significant differences between the sexes at $P < 0.05$ according to independent-samples t test (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns, not significant)

Flowering phenology (including the flowering phase) is very sensitive to temperature, particularly during the spring months (Osborne et al. 2000; Craufurd and Wheeler 2009), and is accordingly considered to be a reliable indicator of climate change. In this regard, previous studies reported that elevated temperature and CO₂ significantly increase the duration of the reproductive phase in *Andropogon gerardii* Vitman, *Dichantheium oligosanthes* ssp. *scribnerianum*, *Hesperostipa comata* (Elias.) Barkworth, *Koeleria macrantha* (Ledeb.) Schult, *Panicum virgatum* L., and *Sphaeralcea coccinea* (Nutt.) Rydb. (Sherry et al. 2007; Reyes-Fox et al.

2014), which is consistent with our findings. Given that changes in flowering times and phase may affect pollination success and synchrony (Fitter and Fitter 2002; Rawal et al. 2015), an extended flowering phase for males would increase pollen dispersal, enhance the attraction of pollinators, and reduce the adverse effects of warming on reproductive success (Glaetli and Barrett 2008; Bandera and Vilagines 2013; Høye et al. 2013). Hence, we conclude that a longer flowering phase in male inflorescences induced by elevated temperature and CO₂ may benefit female flowers by increasing the receipt of pollen, thereby improving pollination efficiency and promoting the reproductive success of *M. alba* L.

Increased temperature and elevated CO₂ had discernible effects on the morphological traits of inflorescences and resulted in different responses between two sexes in mulberry in the present study, which indicate that elevated temperature and CO₂ treatments have differential sex-dependent effects on flower morphology. According to Charlesworth and Charlesworth (1981) and Worley and Barrett (2000), sexual differences could be the result of different trade-offs in resource demanding and allocation, such as nitrogen and carbon in flowers. Female is more carbon-demanding than male in flower because of seed and fruit development (McDowell et al. 2000), whereas male is more nitrogen-demanding than female because of pollen production (Harris and Pannell 2008). Therefore, increased temperature and elevated CO₂ concentration could indirectly affect floral development by affecting carbon or nitrogen contents of flower. Consistent with our conjecture, our results provide experimental evidence that nitrogen content in female and male flowers was significantly changed under elevated temperature or CO₂ concentration treatments. On the other hand, our results in C/N ratio

Table 3 Correlation coefficients among morphological characters, the concentrations of carbon (C) and nitrogen (N), and the ratio of carbon (C) to nitrogen (N) in female (upper triangle) and male (lower triangle, italic) flowers from *Morus alba* L.

	C	N	C/N	IN	IL	NFPI	FIW	FFW	TL	TW	OL	OD
C		-0.005	0.100	-0.522*	0.046	-0.142	0.023	0.127	0.041	0.013	0.291	0.188
N	0.690**		-0.995**	0.385	-0.380	-0.386	-0.458	-0.144	-0.546*	-0.444	-0.134	-0.174
C/N	-0.562*	-0.985**		-0.442	0.388	0.375	0.464	0.160	0.554*	0.450	0.166	0.197
IN	0.542*	0.679**	-0.637**		-0.377	-0.250	-0.369	-0.255	-0.531*	-0.407	-0.410	-0.428
IL	0.565*	0.663**	-0.597*	0.770**		0.866**	0.811**	0.702**	0.654**	0.514*	0.561*	0.628**
NFPI	0.664*	0.742**	-0.676**	0.805**	0.855**		0.789**	0.533*	0.588*	0.472	0.402	0.409
FIW	0.692**	0.764**	-0.695**	0.818**	0.924**	0.941**		0.763**	0.867**	0.839**	0.704**	0.635**
FFW	0.428ns	0.472ns	-0.442ns	0.570*	0.552*	0.509*	0.657**		0.799**	0.700**	0.893**	0.893**
TL	0.383ns	0.459ns	-0.432ns	0.457ns	0.407ns	0.479ns	0.431ns	0.575*		0.898**	0.823**	0.769**
TW	-0.086ns	-0.095ns	0.081ns	-0.079ns	-0.109ns	-0.160ns	0.019ns	0.335ns	-0.185ns		0.708**	0.672**
AL	0.261ns	0.382ns	-0.385ns	0.443ns	0.255ns	0.470ns	0.483ns	0.789**	0.482ns	0.365ns		0.841**
AW	-0.074ns	0.066ns	-0.122ns	0.061ns	-0.114ns	0.030ns	0.107ns	0.556*	-0.053ns	0.363ns	0.641**	

C/N the ratio of carbon (C) to nitrogen (N), IN inflorescence number, IL inflorescence length, NFPI no. of flowers per inflorescence, FIW fresh inflorescence weight, FFW fresh flower weight, TL tepal length, TW tepal width, OL ovary length, OD ovary diameter, AL anther length, AW anther width. Significance is indicated as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant.

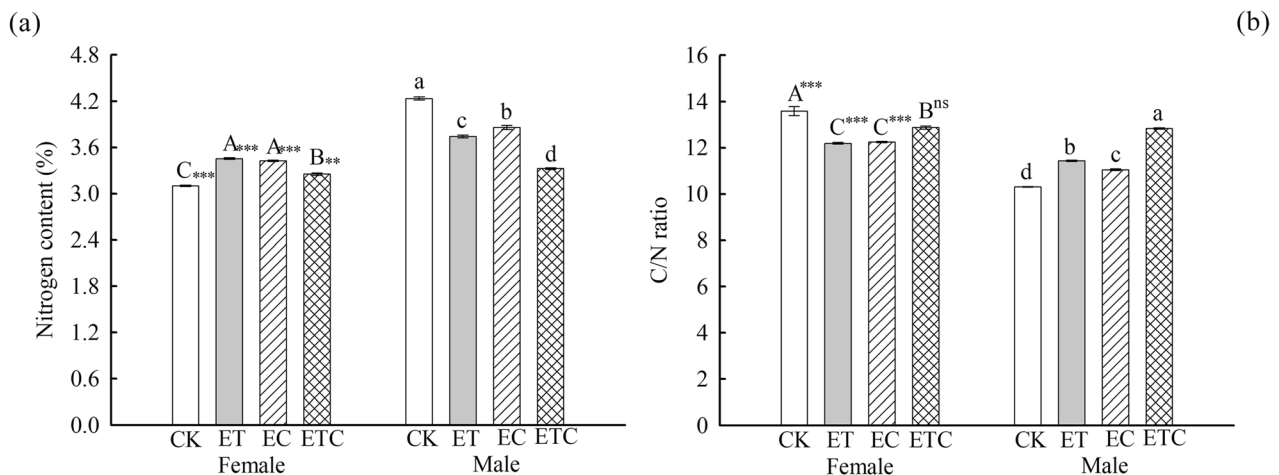


Fig. 6 The effects of elevated temperature, elevated CO₂ concentration, and their combination on nitrogen content and C/N ratio in *Morus alba* L. CK control, ET ambient + 2 °C, EC ambient + 380 ppm, ETC combined ET/EC conditions. Different letters above bars for the same sex group indicate significant differences among treatments according to one-way

ANOVA followed by Duncan's test ($P < 0.05$). Asterisks above bars denote statistically significant differences between the sexes at $P < 0.05$ according to independent-samples t test (** $P \leq 0.01$; *** $P \leq 0.001$; ns not significant)

(significantly negatively correlated with male inflorescences, but was positively correlated with tissue of female flowers) suggest that a lower carbon (or higher nitrogen) level is beneficial for flower development. This is consistent with previous studies that an adequate level of nitrogen results in larger and more numerous inflorescences in *Calluna vulgaris* (L.) Hull, *Canarium album* L., and *Cucurbita maxima* Var. "Little Cutie" (e.g., Gordon et al. 1999; Fernandez-Escobar et al. 2008; Hoover et al. 2012). Moreover, significant negative correlations between the C/N ratio and male inflorescence traits also indicate that more nitrogen for pollen production may have restricted the development of male inflorescences. For female flower, high C/N ratio is positively related with tepal length, which suggests that female flower tend to increase carbon investment in tepal extension to protect ovary. This may be a crucial strategy of ecological adaptability in successful pollination.

In addition, concomitant with an increase in the number of female inflorescences, we observed a decrease in the number of male inflorescences on *M. alba* L. plants. According to Galen (1999), small flowers could reduce the physiological stress associated with reproduction in times of resource limitation. Smaller and fewer male flowers (or inflorescences) were produced under elevated temperature and CO₂ concentration may have been due to the increased allocation of resources to female flowers under these conditions. In this regard, increasing the number of female inflorescences, and thereby by increasing the probability of receiving pollen, could be a more effective strategy in terms of enhancing pollination efficiency than producing larger flowers (or inflorescences) (Ohara and Higashi 1994). It is still needed to investigate the sex differentiation mechanism in

molecular (e.g., RNA, DNA methylation, and histone modification) and in evolution.

5 Conclusions and future perspectives

In terms of flower number, biomass, and morphological traits, the results of this study provide evidence that the male inflorescences of *M. alba* L. are more negatively affected than are female inflorescences in response to elevated temperature, elevated CO₂ concentration, and a combination of elevated temperature and CO₂. Additionally, we found that the C/N ratio was negatively associated with the morphological characters of male inflorescences, whereas it was positively associated with tepal length in female flowers. Our findings suggest that floral morphological traits and sex differentiation of mulberry flowers would be affected by global warming and result in changing the sex ratio. In this regard, the present findings may have significant implications for the optimization of fertilization management designed to regulate inflorescence number and morphology in mulberry under conditions of global warming.

Data availability statement The datasets generated and/or analyzed during the current study are available in the Zenodo repository (Li et al. 2019) at <http://doi.org/10.5281/zenodo.3402313>.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Appendix

Fig. 7 The effects of elevated CO₂ concentration, elevated temperature, and their combination on the growth and development of *Morus alba* L. after 18 months growth.



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