Sex and flowers: testing the resource-dependent selection hypothesis for flower sex allocation

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ABSTRACT

Context. Monoecious plants can adjust their proportional investment in male and female flowers to maximise reproductive fitness. The female reproductive function (seeds) often has greater resource costs than the male (pollen). Larger plants are generally thought to have greater resource availability and should have a female biased sex ratio, referred to as the size-dependent selection hypothesis. However, empirical tests of this hypothesis have found mixed support. This may be because size alone is not always a reliable proximate value for resource availability, which can be influenced by other abiotic factors. **Aims.** *Breynia oblongifolia* (Phyllanthaceae) is a perennial monoecious plant with unisexual moth-pollinated flowers from eastern Australia. Fruit production in *Breynia* is heavily influenced by rainfall, which is highly variable. We hypothesised that where soil moisture limits female function, *Breynia* would produce more male flowers (i.e. resource-dependent selection). **Methods.** We used a multi-year observational dataset to look for evidence of resource-dependent flower sex ratios in a wild population and conducted a manipulative glasshouse experiment to test alternative hypotheses for flower sex selection. **Key results.** In both our manipulative glasshouse experiment and observed wild population, decreasing soil water content resulted in higher proportions of male flowers, supporting the resource-dependent sex selection hypothesis. **Conclusions.** Soil moisture influences flower sex ratios but plant size does not. **Implications.** Future studies should not assume that height equates to resource wealth, as this is often overly simplistic and ignores the potential for key resources, like soil moisture or light, to fluctuate.

Keywords: *Breynia oblongifolia*, flower sex selection, monoecious, Phyllanthaceae, plant height, resource-dependent sex selection, size-dependent sex selection, soil moisture.

Introduction

The female reproductive function of a plant often has different resource needs than the male (Lloyd and Webb 1977; Korpelainen 1994; Abe 2002; Zhang and Jiang 2002; Shwe *et al.* 2020). Intraspecies studies have shown that relative investment in female flowers and female structures within flowers (i.e. ovules) increases with plant size (Lloyd and Bawa 1984; Clay 1993; Klinkhamer *et al.* 1997; Wright and Barrett 1999; Méndez and Traveset 2003; Andrieu *et al.* 2007; Reekie and Bazzaz 2011; Zhang, Zhu *et al.* 2014; Vélez-Mora *et al.* 2021). This is because the production of fruits and seeds requires additional resources after the production of flowers (Lloyd and Webb 1977; Nicotra 1999). Larger plants generally have more resources available to them and, as such, are likely to allocate more of these resources to female flowers, or ovules in the case of plants with hermaphroditic flowers (Méndez and Traveset 2003). This is referred to as the size-dependent selection hypothesis (Lloyd and Bawa 1984; Clay 1993; Klinkhamer *et al.* 1997; Liu *et al.* 2009; Shwe *et al.* 2020).

The size-dependent selection hypothesis does not explicitly predict that larger plants should have a greater proportion of female flowers. In some plants, there is greater allocation of resources to male flowers with increasing plant size (Ishii 2004; Liao and Zhang 2008; Delesalle and Mazer 2009; Liu *et al.* 2009; Wang *et al.* 2019). For example,

in wind-pollinated plants, the relative investment in male flowers and pollen often increases in larger trees (Ackerly and Jasieński 1990; Bickel and Freeman 1993; Pannell 1997). This may be because the male function is more costly in wind-pollinated plants, e.g. when producing large quantities of pollen (Klinkhamer *et al.* 1997). As such, the size-dependent selection hypothesis has been used to explain male and female biased sex ratios under a broad variety of plant pollination systems.

Plant size is not the only factor that can affect floral sex allocation. Size is often strongly correlated with plant age (Wright and Barrett 1999), which can itself have important effects on reproductive effort (Roach 1993; Sherman *et al.* 2019) and the relative investment in male and female reproduction (Ramírez and Davenport 2016). Allocation to male flowers has also been shown to change with altitude, and not plant size, in a study of bumblebee pollinated *Pedicularis* spp. (Orobanchaceae) (Guo *et al.* 2010; Zhang *et al.* 2011). It has been suggested that the increases in allocation to male flowers may be due to pollen limitation in these high altitude plant communities. Indeed, pollen limitation is believed to be one of the primary factors in promoting the evolution of separate unisexual flowers (Crowley *et al.* 2017). Plants may also favour the production of seeds over pollen for other ecological reasons. For example, some plants have been shown to allocate more resources to female flowers (and seeds) under semi-arid climates, which may result in increased reproductive success under these hostile conditions (Teixido and Valladares 2019). Breeding systems can also affect resource allocation, with facultatively outcrossing species (xenogamy) allocating a greater proportion of biomass to male function than plant species that self-pollinate (autogamy) (Cruden and Lyon 1985). Relative investment in male and female resource allocation can also have a strong phylogenetic signal, which must be accounted for in interspecies studies (Teixido *et al.* 2017). Taken together, it is clear that many other factors in addition to plant size can influence the relative investment in male and female structures in flowering plants.

Many studies have found no differences in the relative proportions of male and female flowers with increasing plant size (Hibbs and Fischer 1979; Matsui 1995; Méndez and Traveset 2003; Vallejo-Marín and Rausher 2007; Han *et al.* 2011; Torices and Méndez 2011; Shwe *et al.* 2020). Furthermore, the effect of increasing plant size on flower sex ratio can vary between populations of the same plant species (Cao and Kudo 2008). The lack of support for the size-dependent selection hypothesis in some studies may be related to the underlying assumption that size reflects resource wealth. This is because resource limitation could involve one or more factors including soil moisture, nutrients and sunlight. The availability of these resources can vary between populations and growing seasons (Zimmerman and Aide 1989; Schlessman 1991;

Korpelainen 1998), making size alone an unreliable predictor of the resources available to a plant at any given time.

Several studies have looked directly at resource limitation for explaining variation in flower sex ratio. Consistent with resource-dependent selection, these studies found that the proportion of male flowers increased with resource limitation (Dorken and Barrett 2004; Han *et al.* 2011; Zhang, Zhang *et al.* 2014). Plants growing at high densities show a higher proportion of male flowers than those growing at low densities, suggesting that competition and associated resource shortages can also drive flower sex ratios (Ackerly and Jasieński 1990; Pannell 1997; Dorken and Pannell 2008; Vélez-Mora *et al.* 2021). Several studies have shown that water-limited plants produce fewer fruits and more male flowers (Barker *et al.* 1982; Wolfe and Shmida 1995, 1997; Geber *et al.* 1999). Indeed, light, nutrient and water stress are all often associated with increases in male flowers in plants (Freeman *et al.* 1976, 1980; McArthur 1977; Hibbs and Fischer 1979; Lloyd and Bawa 1984; Zimmerman 1991; Korpelainen 1998; Ortiz *et al.* 2002). Theoretical models suggest that such labile sex expression would be strongly adaptive under variable environmental conditions, where certain conditions favour male or female reproduction (Freeman *et al.* 1976, 1980; Charnov and Bull 1977). Many studies have found evidence that is consistent with a resource-dependent selection hypothesis. However, no studies have directly compared the importance of plant size and resource limitation in flower sex selection.

Monoecious plants bear separate male and female flowers on the same individual plant. This separation of male and female flowers allows plants the flexibility to adjust their resource investment in male (pollen) and female (seed) reproduction (Lloyd 1972; Delesalle 1992; Fox 1993; Costich 1995; Korpelainen 1998; Sarkissian *et al.* 2001; Dorken and Barrett 2003). If the relative fitness of one sexual function, either pollen or seeds, is limited by an external factor, monoecious plants can invest in the other function to increase the total number of offspring produced (Freeman *et al.* 1981; Moore and Pannell 2011). For example, if fruit production (the female reproductive function) is limited by a key resource, such as light or soil water availability, then plants can still sire offspring on other plants by investment in male flowers and pollen. As such, individual monoecious plants can respond to environmental variability by adjusting their flower sex ratio to maximise reproductive fitness (Ghiselin 1969; Charnov 1982; Lloyd and Bawa 1984; Iwasa 1991; Wright and Barrett 1999).

Breynia oblongifolia (Phyllanthaceae) (Fig. 1), henceforth *Breynia*, is a common perennial woody shrub that is native to the *Eucalyptus*-dominated forests of eastern Australia; it is monoecious with separate (unisexual) male and female flowers that are pollinated exclusively by at least two highly specific species of *Epicephala* moth (Gracillariidae),
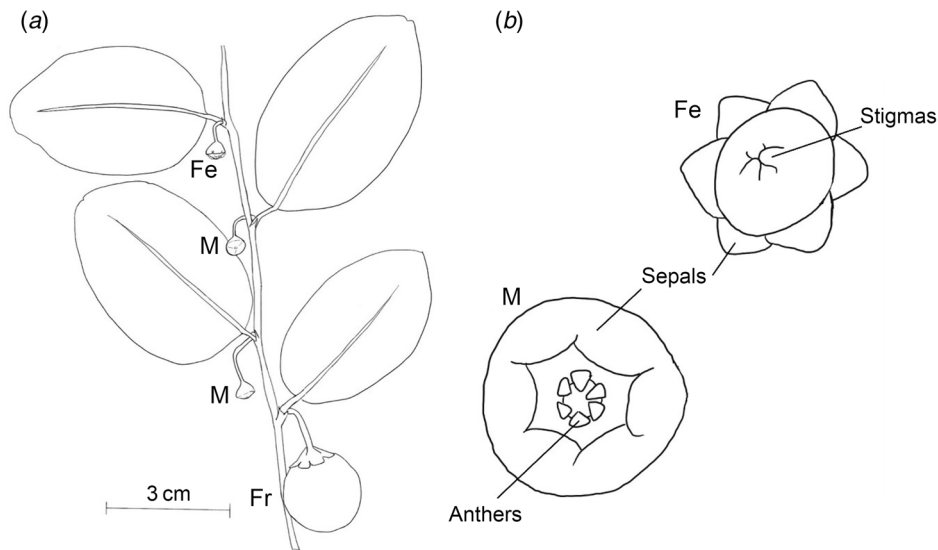
Fig. 1. (*a*) The typical arrangement of male flowers (M), female flowers (Fe) and fruits (Fr) on *Breynia oblongifolia* (Phyllanthaceae). Diagram modified from Finch *et al.* (2021*b*) and used with permission of the authors. (*b*) A magnified view of female and male flowers showing the reproductive structures. Male flowers have anthers that are strongly enclosed by sepals. Female flowers have three highly reduced stigmas that converge towards the centre of the flower.

known only as *Epicephala* sp. A and B (Finch *et al.* 2018, 2019, 2021*a*). Rainfall is highly variable in eastern Australia (Ashok *et al.* 2003; Risbey *et al.* 2009) and often limits plant growth (Bernacchi and VanLoocke 2015). Previous investigations have shown that *Breynia*'s flowering and fruiting is strongly influenced by precipitation (Finch *et al.* 2021*a*). Because of this, we reasoned that the most limiting resource to fruit production in *Breynia* is the availability of soil moisture. We hypothesised that where female function is limited by soil moisture availability, plants will invest in a greater proportion of male flowers (i.e. resource-dependent selection). This hypothesis was tested in two ways. Firstly, we used a pre-existing multi-year observational dataset to look for evidence of resource-dependent flower sex ratios in *Breynia*. Secondly, we conducted a manipulative soil moisture experiment to test whether the resource-dependent hypothesis influences flower sex ratio in *Breynia* under controlled conditions. By measuring plant size, this experiment also allowed us to look for evidence of size-dependent sex selection. By testing these hypotheses, we hope to better understand the drivers of floral sex ratio in monoecious flowering plants.

Materials and methods

Study system

Phyllanthaceae (Malpighiales) is a morphologically diverse family of mainly tropical species that includes *Phyllanthus*, one of the largest genera of flowering plants

(~1200 species). *Phyllanthus sensu lato* also includes several large genera or sub-genera that are phylogenetically nested within *Phyllanthus* but have yet to be formally renamed, such as the genus *Breynia* (Hoffmann *et al.* 2006; Hidalgo *et al.* 2020).

B. oblongifolia (Fig. 1), henceforth *Breynia*, is woody perennial shrub native to eastern Australia. *Breynia* is monoecious and can become reproductively active at a young age, producing flowers and fruit from ~1 to 2 years old (Finch *et al.* 2021*a*). However, fruit are only produced in the presence of the plants specialist moth pollinators (Finch *et al.* 2021*a*). Female flowers are usually present throughout the year but decline in number over the winter, and during dry periods, when they are often pollinated but dormant (Finch *et al.* 2021*a*). Male flowers are only present during the spring and summer and are often absent during very hot and dry weather. The abundance of both male and female flowers is significantly predicted by recent precipitation, with the number of both male and female flowers increasing rapidly following rainfall (Finch *et al.* 2021*a*).

Field observations

To test the resource limitation hypothesis, we analysed a pre-existing dataset of *Breynia* floral phenology (Finch *et al.* 2021*a*) and combined it with soil moisture data from a long running forestry experiment occurring at the same site. EucFACE is a free-air carbon enrichment experiment, set in a remnant patch of endangered Cumberland Plain Woodland, which is owned and operated by the Hawkesbury Institute for

the Environment, Western Sydney University in New South Wales (NSW), Australia (Ellsworth *et al.* 2017). *Breynia* makes up a large component of the woodland understory at the EucFACE site. Twenty plants were chosen by walking along two random transect lines that ran outside of the carbon enrichment rings (>15 m distant) and choosing the nearest plant >1 m in height every 10 m. Four branches, ~30 cm long, were selected on the four cardinal points of each plant and marked for monitoring by repeat survey. Plants were surveyed every 2–4 weeks between September 2015 and April 2018. Less frequent surveys were undertaken in the winter months when plants were dormant and the number of flowers low and stable. On each visit, the numbers of male and female flowers were counted on each branch and later summed by plant. In total, we conducted 34 surveys of flowering phenology. Because female flowers are often present but functionally dormant (Finch *et al.* 2021*a*, 2021*b*), we used the presence of male flowers to determine flowering events, such that any plant with more than one male flower was categorised as flowering. For each flowering event, we calculated the proportion of male to female flowers using the survey with the maximum number of male flowers for each plant (i.e. peak flowering). *Breynia* plants at the site underwent four distinct mass flowering events during our 2 years of phenology surveys (Fig. 2*a*).

At the EucFACE site, a network of six Thetaprobe ML2x soilmoisture sensors (Eijkeljamp Agrisearch Equipment, Giesbeek, Netherlands) collect measurements of volumetric soil water content (VWC) from within the rings at 5, 30 and 75 cm below the surface every 15 min. All moisture probes were at least 5 m from the surveyed *Breynia* plants. Because no moisture probes were sufficiently close to get reliable values for individual plants, we averaged soil moisture readings across all depths and probes to determine the average volumetric soil water content across the entire site per day. The average daily soil moisture readings were then matched by date to our phenology surveys. Soil moisture levels on the day of each survey are likely to be less relevant to flowering phenology than soil moisture levels in the previous days and weeks. However, calculating the most appropriate time frame to use for our phenology surveys is beyond the scope of this study. Regardless, we believe that soil moisture values on the day of each survey should give a suitable approximate value for the conditions experienced by plants at the site in the previous weeks and months.

Controlled environment experiment

The wild population provided evidence supporting the resource-dependent selection hypothesis. However, our re-purposed dataset did not include measurements of plant size and cannot be used to test the size-dependent sex selection hypothesis. As such, we conducted a second
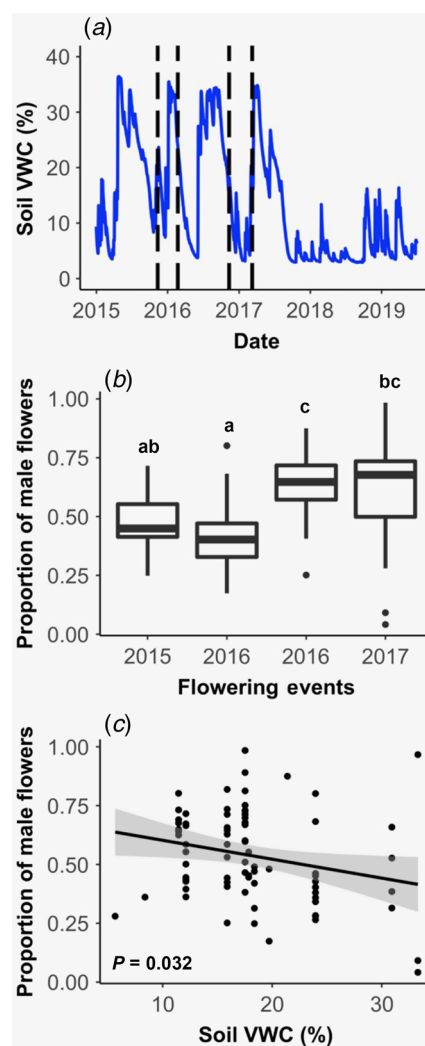
Fig. 2. (*a*) Soil Volumetric Water Content (%) (VWC) at the EucFACE field site in Richmond, NSW, Australia, during the period of study. The flowering events from (*b*) are annotated as dashed black lines. The site experienced a pronounced drought between 2018 and 2020 but unfortunately no phenology surveys were undertaken during this time. (*b*) Median proportion of male flowers in 20 *Breynia oblongifolia* individuals across four successive flowering events between September 2015 to October 2017 at the EucFACE site. Boxes with any common letter are not significantly different (*P* > 0.05). Box plots represent the median, upper and lower quartiles. (*c*) The relationship between soil volumetric water content and the proportion of male flowers across the four flowering events.

experiment under controlled conditions that allowed us to test both the size-dependent and resource-dependent hypotheses. In August 2019, we purchased 75 *Breynia* seedlings from Indigo Native Nursery (Sydney, NSW, Australia). All seedlings were ~5 cm tall and were of very similar age, having been sown at the same time by nursery staff, although the exact date of sowing is not known. The seedlings were maintained in their original 200-mL 'tubestock' containers for 1 year and were watered daily

using an overhead spray irrigation system. In the spring of 2020, all 60 surviving seedlings were re-potted into 1.3-L plastic pots using Australian Native Soil Mix (Turtle Landscape Supplies, South Windsor, NSW, Australia). At this point plants were ~2 years old. After re-potting, all plants were moved into a climate-controlled glasshouse maintained at 24°C and 50% RH. Plants were measured and ranked by height (soil surface to apical meristem) before being alternately sorted between two large plastic watering trays corresponding to our two treatment groups: high soil moisture and low soil moisture. Plants were randomly allocated to their positions within the experimental design. Each tray contained 30 plants. All plants received 1 g of Scotts Osmocote Native Slow Release Fertiliser (Scotts Miracle-Gro Company, Marysville, OH, USA) shortly after re-potting. Plants in the high soil moisture treatments received 360 mL of water every 3 days, whereas plants in the low soil moisture treatment received 180 mL every 3 days. Watering regimes were chosen to simulate the soil moisture levels observed in wild populations (Fig. 2a), with a soil VWC of 10–30%.

Volumetric soil moisture was quantified using an Acclima SDI-12 Sensor Reader (Acclima, Meridian, ID, USA) to measure five randomly selected plants from each treatment group every 14 days, before and after watering. Measurements were taken according to manufacturer's instructions. The two watering regimes resulted in significantly different soil moisture levels between the two treatments ($F = 131.7$, d.f. = 1, $P < 0.001$). The average soil moisture content in the higher water treatments was 21.3% (s.d. = 5) before watering and 32.4% (s.d. = 7.2) after watering. Average soil moisture content in the low water treatment was 7.9% (s.d. = 4.9) before watering and 19.8% (s.d. = 9) after watering (Fig. S1).

During the experiment, several plants became infested by an unidentified species of aphid, likely to be *Schoutedenia lutea* (Tomiuk *et al.* 1991). Infestations were treated with spot application of Yates 750 mL Ready To Use Pyrethrum Insecticide (DuluxGroup pty ltd, Melbourne, Vic., Australia) and three subsequent releases of 500 green lacewing larvae (*Mallada signatus*) to act as biological control agents (Bugs For Bugs, Toowoomba, Qld, Australia). The infected plants showed no obvious signs of damage or distress as a result of aphid feeding and were thereafter included in the experiment.

The number of male and female flowers on each plant was counted every 14 days for 2 months. In November, the height of each plant was measured again, and growth was calculated as the difference between the second and first height measurements. All above ground biomass was harvested manually with secateurs, placed in paper bags and dried at 70°C in a D2400 drying oven (Steridium Pty Ltd, Australia) for 5 days and then weighed using a QM-7264 1KG Precision Scale (Digitech Pty Ltd, Australia).

Statistical analysis

All statistical analyses were conducted in RStudio (ver. 1.0.153, RStudio PBC, Boston, MA, USA, see https://rstudio.com/), using R (ver. 1.414, R Foundation for Statistical Computing, Vienna, Austria, see https://www.r-project.org/). For all our analyses, the proportion of male flowers was calculated as the number of male flowers ÷ total number of male and female flowers per plant. The proportion of male flowers in our field observations was not significantly different from a normal distribution (Shapiro–Wilk normality test, $W = 0.986$, $P = 0.59$). A one-way analysis of variance (ANOVA) test was used to determine whether there was a significant difference in the proportion of male flowers across the four flowering events. A generalised linear mixed model (GLMM) was then used to test for the effect of soil moisture on the proportion of male flowers in our field observations, using the *nlme* library (ver. 3.1-158, Pinheiro, Bates and the R Core Team, see https://CRAN.R-project.org/package=nlme; Pinheiro and Bates 2000) and specifying an 'REML' fitting method. Because our counts of male flowers came from 20 individual plants repeated across four flowering events (Fig. 2a), we specified plant identity as a random factor in order to account for variation between individuals arising from differences in soil profile, light levels, age, etc.

In our controlled environment experiment, the proportion of male flowers did not follow a normal distribution (Shapiro–Wilk normality test, $W = 0.9$, $P < 0.001$). To account for this, we used generalised linear models (GLMs) in the R stats package (ver. 3.6.3, R Foundation for Statistical Computing) to determine if the proportion of male flowers varied as a function of soil water availability (factor: high or low), plant size and their interactions, specifying a binomial error distribution (link = logit). We used two measures of plant size; plant height as measured at the end of experiment (cm) and dry biomass (g) after harvesting and drying. Plant height and biomass were highly correlated (cor = 0.80, $t = 9.7$, $P < 0.0001$) but are both used as measures of plant size within the literature. To avoid issues with autocorrelation, we constructed two GLM models as above, using both height and biomass as separate measures of plant size. We constructed another GLM to model the effect of our soil water availability treatment on the total number of flowers per plant. Counts of total flowers did not follow a normal distribution (Shapiro–Wilk normality test, $W = 0.9$, $P < 0.001$) and were analysed using a GLM with a poisson error distribution. We also tested whether plants in our high and low soil water availability treatments had grown significantly more during the experiment. To do this we tested if growth, the difference between the initial and final plant height measurements, and final biomass differed significantly between treatments. Measurements of growth followed a normal distribution (Shapio–Wilk normality test, $W = 0.98$,

$P = 0.83$), and were analysed using a one-way ANOVA with soil water availability as the only explanatory variable. Measurements of final biomass did not follow a normal distribution (Shapiro–Wilk normality test, $W = 0.92$, $P = 0.0012$) and were analysed using a GLM with a poisson error distribution.

Results

Field observations

Our wild population allowed us to test the extent to which the resource-dependent hypothesis holds true over multiple growing seasons in naturally occurring *Breynia*. From September 2015 to October 2017, *Breynia* plants at the EucFACE field site underwent four distinct mass flowering events (Fig. 2a). The proportion of male flowers was found to be significantly different between the four flowering events ($F = 6.12$, d.f. = 3, $P < 0.0001$) (Fig. 2b). Analysis of the GLMM showed that increasing soil moisture had a significant negative effect on the proportion of male flowers (Est. = -0.72, s.e. = 0.33, d.f. = 58, $t = -2.2$, $P = 0.032$) (Fig. 2c).

Controlled environment experiment

Consistent with the resource-dependent selection hypothesis, there was a significant difference in the proportion of male flowers between the treatment groups, with drier plants being significantly more male ($z = 5.99$, d.f. = 1, $P < 0.00001$) (Fig. 3a). Plant size had no significant effect on the proportion of male flowers regardless of whether size was measured as final plant height ($P = 0.276$) or final dry biomass ($P = 0.76$) (Fig. 4a, b). There was also no significant interaction between either height ($P = 0.71$) or biomass ($P = 0.74$) and soil moisture availability. Plants in the low soil moisture treatment had fewer total flowers ($z = -7.85$, $P < 0.0001$) (Fig. 3b). Plants in the low soil moisture treatment also grew significantly less than plants in the high soil moisture treatment ($F = 4.8$, d.f. = 1, $P = 0.031$) (Fig. 3c) and had significantly lower final dry biomass ($z = -6.1$, d.f. = 1, $P < 0.00001$) (Fig. 3c).

Discussion

We tested two hypotheses on flower sex selection in *B. oblongifolia*. Our results support the resource-dependent selection hypothesis, as decreasing soil moisture increased the proportion of male flowers. This was true of both our wild population and of plants grown under controlled conditions. Our results are consistent with the view that where female reproduction is limited by a lack of resources (here, soil moisture), monoecious plants can adapt by
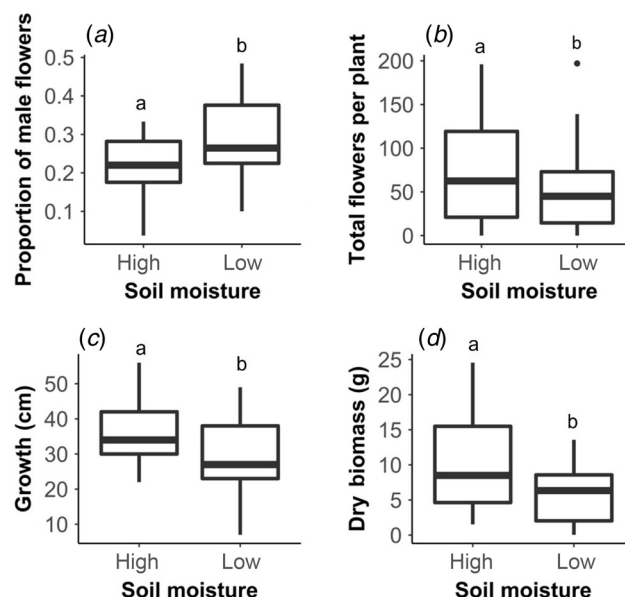
Fig. 3. The median (a) proportion of male flowers, (b) counts of all flowers (c) plant growth (change in height from start to end of the experiment) and (d) post-harvest dry biomass (g) in *Breynia oblongifolia* ($n = 60$) grown under high and low soil moisture conditions for eight weeks. Boxes with any common letter are not significantly different. Box plots represent the median, upper and lower quartiles.

shifting their investment towards male flowers to maximise their reproductive fitness (Ghiselin 1969; Charnov 1982; Lloyd and Bawa 1984; Iwasa 1991; Wright and Barrett 1999).

The size-dependent selection hypothesis suggests that larger plants should have a greater proportion of female flowers (Lloyd and Bawa 1984; Clay 1993; Klinkhamer et al. 1997; Liu et al. 2009; Shwe et al. 2020). No such trend was evident in our data. Plants in our high soil moisture treatment grew more and had greater biomass than those grown in our low soil moisture treatment. Despite this, and in contrast to many previous studies (Méndez and Traveset 2003; Andrieu et al. 2007; Zhang, Zhang et al. 2014; Vélez-Mora et al. 2021), we found no evidence to suggest that plant size (both height and biomass) directly influences the proportion of male flowers in *Breynia*. Our analysis showed that there is no direct effect of plant size (height or biomass) on the proportion of male flowers, despite high variation in both. This can be seen in clearly in Fig. 4, where plants of a similar height or biomass generally have a higher proportion of male flowers when grown under dry conditions. Our results are consistent with previous research that suggest that flower sex allocation is independent of plant size and is in fact determined by environmental and ecological conditions (Korpelainen 1998; Nanami et al. 2004; Guo et al. 2010; Zhang et al. 2011).
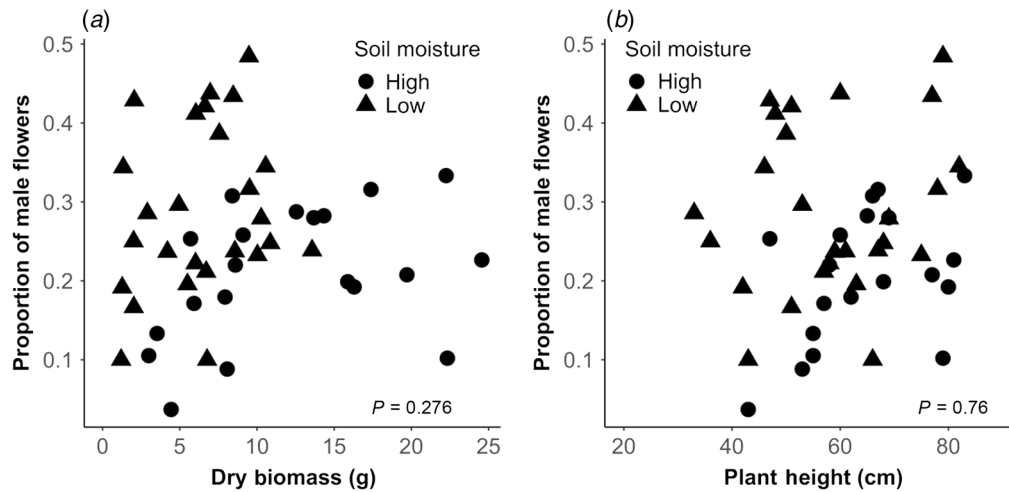
Fig. 4. The relationship between (*a*) final plant biomass and (*b*) final plant height with the proportion of male flowers in *Breynia oblongifolia* (*n* = 60) grown under high and low soil moisture conditions for 8 weeks.

Limitations of field observations

The proportion of male flowers in our wild *B. oblongifolia* population generally increased over time. Our analysis showed that this change was driven by decreases in soil moisture at the site. This point illustrates the potential pitfalls of using proximate values for resource wealth (i.e. plant size). Future studies using wild populations should not simply assume that height equates to resource wealth (Lloyd and Bawa 1984; Clay 1993; Klinkhamer *et al.* 1997; Korpelainen 1998), as this is often overly simplistic and ignores the potential for key resources, like soil moisture or light, to fluctuate. Instead, studies should first attempt to identify which resources are most limiting to male and female reproduction. Identifying the most limiting resource will result in better, more precise hypotheses and experiments in studies of sex resource selection, and lower chances of returning false negative results.

Our original dataset of flower sex selection in the wild population at EucFACE did not include data on plant height or biomass. Because of this, it was not possible to determine the effect of plant size on the proportion of male flowers in this wild population. In the future it would be interesting to see if the greater size variation in these populations does influence the proportion of male flowers. Attempts could also be made to quantify the availability of soil nutrients and light, which we were not able to do. Determining the relative importance of different resources, with a greater range of plant sizes, would be a critical test of the resource-dependent selection hypothesis.

Limitations of the controlled environment experiment

Although we found a significant relationship between soil moisture and floral sex selection, substantial variation in

the proportion of male flowers occurred in both experiments. It appears that factors other than soil moisture may also have affected flower sex selection in our study. Pollen limitation, decreasing photoperiod, low illumination, reduced nutrient availability and extreme temperatures can all increase the proportion of male flowers or male structures (anthers or pollen) in plants (Dodson 1962; Gregg 1973; McArthur 1977; Hibbs and Fischer 1979; Freeman *et al.* 1980; Matsui 1995; Korpelainen 1998; Guo *et al.* 2010; Zhang *et al.* 2011). In our controlled environment experiment, we provided an equal amount of nutrients to all plants and kept them under controlled climatic conditions. All plants are also likely to be equally pollen limited as *Breynia*'s highly specific pollinators (Finch *et al.* 2018, 2021*b*) could not access the plants in the glasshouse. Furthermore, our experiment was conducted during the spring and early summer under long duration photoperiods. Because of this, we believe that the variability seen in the glasshouse study is unlikely to be explained by either nutritional differences, changing photoperiod or climatic extremes.

In our glasshouse experiment, plants were randomly allocated to their positions within the experimental design. However, we made no attempt to standardise the photosynthetic light available to each plant. It is therefore possible that some plants may have experienced differing levels of illumination because of their position within the glasshouse. Furthermore, towards the end of the experiment some plants were roughly twice as tall as the smallest individuals (Fig. 4*b*), which could have created significant shading for smaller plants. Wild populations of *Breynia* are also subject to varying levels of illumination due to differences in the tree canopy and shading from conspecifics (J. T. D. Finch, pers. obs.). Varying light levels could explain the high variation seen in the proportion of male flowers

across both the wild population and glasshouse experiment (Dodson 1962; Gregg 1973). Damage by insects and other forms of physical trauma have also been linked to changes in sex expression in flowering (Freeman *et al.* 1980; Korpelainen 1998; Blake-Mahmud and Struwe 2020). As such, the aphids present on some plants during the experiment may also have affected the sex ratio of infested plants. Regardless of the cause, our results illustrate the broad range of potential factors that can influence sex expression in flowering plants.

An important caveat to our results is the plants used in our glasshouse study are relatively young and small compared to wild populations. In our glasshouse study, all plants were ~2 years old and between 30 and 90 cm tall. In the wild, mature *Breynia* are usually ~2 m tall (J. T. D. Finch, pers. obs.). The age of these wild populations is unknown, but is likely greatly exceed 2–3 years. Nevertheless, *Breynia* plants are reproductively active and can produce large numbers of viable fruits from ~2 years old (Finch *et al.* 2021*b*). Furthermore, given that the younger glasshouse grown plants responded similarly to older wild plants to low soil water availability, we are confident that the results of our glasshouse experiment are indicative of real world populations despite the young age of the plants used.

Underlying mechanisms

Monoecious plants produce both male and female flowers. It is likely that the labile sex ratios seen in monoecious plants are the result of differential regulation of sex determination genes (Irish and Nelson 1989; Korpelainen 1998; Khryanin 2002). Evidence suggest that the regulation of flower sex ratios in plants is mediated by various plant hormones, including cytokinin, gibberellin, auxin, ethylene and abscisic acid (Chailakhyan 1979; Freeman *et al.* 1980; Khryanin 2002; Iqbal *et al.* 2017). In particular, the relative levels of cytokinins to gibberellins has been widely linked to the proportion of male and female flowers, with higher levels of cytokinins being correlated with a greater proportion of female flowers (Freeman *et al.* 1980; Korpelainen 1998; Khryanin 2002). Importantly, the levels of some plant hormones are known to respond to environmental stimuli (Freeman *et al.* 1980). Cytokinins are produced in the plant root system (Khryanin 2002) and under water stress there is reduced transport of this hormone to the rest of the plant (Freeman *et al.* 1980). It is possible that altered levels of cytokinins under low soil moisture conditions may cause the differential expression of sex determination genes in *Breynia*. Proving this, however, would require further experimentation.

Ecological implications

B. oblongifolia is involved in a nursery pollination mutualism with two closely related species of *Epicephala* moth. These highly specific pollinators lay their eggs within female flowers at the time of pollination (Finch *et al.* 2018, 2019, 2021*b*). The pollinator larvae then develop by consuming slightly more than half of the seeds (usually six) within each growing fruit. Usually only a single larva emerges from each fruit, suggesting that moths generally avoid laying multiple eggs to avoid larval competition (Finch *et al.* 2019), although lethal competition between larvae is also a possibility. Flowering and fruiting in *Breynia* is heavily dependent on rainfall and soil moisture (Finch *et al.* 2021*a*), and consequently, the interactions between *Breynia* and its moth pollinators are strongly influenced by climatic effects.

The current study suggests additional mechanisms by which rainfall likely influences this plant–pollinator mutualism. Under dry conditions, *Breynia* plants are likely to increase their proportion of male flowers. Although *Epicephala* moths require male flowers for pollen and potentially nectar (Kawakita and Kato 2004), the moths can only complete their lifecycle in female flowers. Drought stress may limit the availability of female flowers and oviposition sites for female moths. Droughts, such as those experienced in NSW in 2018 and 2019 (Fig. 2*a*), are predicted to increase in both frequency and severity in Australia (Dey *et al.* 2019). The lower availability of female flowers may result in increased competition for oviposition sites both within and between pollinator species (Finch *et al.* 2018 2019). What effect this will have on the mutualism is unknown, but could result in increased rates of egg laying per fruit thereby increasing seed destruction, potentially affecting plant reproductive fitness. As such, how drought and resource-dependent sex selection influence the outcome of the mutualism remains to be seen.

Conclusions

Here, we provide evidence that *B. oblongifolia* conforms with the resource-dependent hypothesis, but not the sizedependent hypothesis. Although we focused on a single species and resource type in this study, we believe our results may be applicable to many monoecious plant species for the following reason. Reproduction in plants is often limited by both pollen deposition and access to available resources (Zimmerman and Aide 1989; Ashman *et al.* 2004; Asikainen and Mutikainen 2005; Knight *et al.* 2006; Rosenheim *et al.* 2016). The limiting resource in question varies between plants, seasons, populations, and ecosystems. For many species, and like *Breynia*, the most limiting resource will be soil moisture (Bernacchi and VanLoocke 2015), but sunlight or nutrients may be the primary limiting factor in other species (Korpelainen 1998). Whereever reproduction is limited by resource availability, plants that can adjust their sex selection are likely to invest more in the least

resource intensive sex. For most animal-pollinated plants, female flowers are more resource intensive than male flowers and thus more likely to be reduced under resource limitation. In this way, plants can respond to environmental variability within their lifetime by adjusting their flower sex ratio to maximise reproductive fitness (Ghiselin 1969).

Supplementary material

Supplementary material is available online.

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