

Could seasonally deteriorating environments favour the evolution of autogamous selfing and a drought escape physiology through indirect selection? A test of the time limitation hypothesis using artificial selection in *Clarkia*

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Received: 26 May 2017 Returned for revision: 15 September 2017 Editorial decision: 23 October 2017 Accepted: 8 December 2017
Published electronically 17 January 2018

- **Background and Aims** The evolution of selfing from outcrossing may be the most common transition in plant reproductive systems and is associated with a variety of ecological circumstances and life history strategies. The most widely discussed explanation for these associations is the reproductive assurance hypothesis – the proposition that selfing is favoured because it increases female fitness when outcross pollen receipt is limited. Here an alternative explanation, the time limitation hypothesis, is addressed, one scenario of which proposes that selfing may evolve as a correlated response to selection for a faster life cycle in seasonally deteriorating environments.
- **Methods** Artificial selection for faster maturation (early flowering) or for low herkogamy was performed on *Clarkia unguiculata* (Onagraceae), a largely outcrossing species whose closest relative, *C. exilis*, has evolved higher levels of autogamous selfing. Direct responses to selection and correlated evolutionary changes in these traits were measured under greenhouse conditions. Direct responses to selection on early flowering and correlated evolutionary changes in the node of the first flower, herkogamy, dichogamy, gas exchange rates and water use efficiency (WUE) were measured under field conditions.
- **Key Results** Lines selected for early flowering and for low herkogamy showed consistent, statistically significant responses to direct selection. However, there was little or no evidence of correlated evolutionary changes in flowering date, floral traits, gas exchange rates or WUE.
- **Conclusions** These results suggest that the maturation rate and mating system have evolved independently in *Clarkia* and that the time limitation hypothesis does not explain the repeated evolution of selfing in this genus, at least through its indirect selection scenario. They also suggest that the life history and physiological components of drought escape are not genetically correlated in *Clarkia*, and that differences in gas exchange physiology between *C. unguiculata* and *C. exilis* have evolved independently of differences in mating system and life history.

Key words: Artificial selection, autogamy, *Clarkia unguiculata*, drought escape, flowering date, herkogamy, life history, mating system, photosynthetic rate, self-fertilization, time limitation hypothesis, water use efficiency.

INTRODUCTION

Mating system evolution is one of the most widely studied subjects in plant reproductive ecology, with a large body of theoretical and empirical work devoted to understanding the causes and consequences of variation in outcrossing rates within and among populations. (Fisher, 1941; Stebbins, 1957; Jain, 1976; Lloyd, 1979; Lande and Schemske, 1985; Barrett and Eckert, 1990; Charlesworth *et al.*, 1990; Holsinger, 1991, 1996; Schoen *et al.*, 1996; Morgan *et al.*, 1997; Eckert *et al.*, 2006; Wright *et al.*, 2013). A major goal of this work has been to identify the factors promoting the transition from outcrossing to selfing within evolutionary lineages, which is thought to occur far more frequently than the reverse, and may well be the most common of all breeding system changes within the angiosperms (Stebbins, 1974; Takebayashi and Morrell, 2001; Charlesworth, 2006; Igic and Busch, 2013).

The evolution of selfing is usually thought to be caused by direct selection on loci influencing the selfing rate when self-fertilizing genotypes have a fitness advantage over outcrossers (Lloyd, 1992). A variety of hypotheses have been proposed to explain the conditions under which this might be the case (Goodwillie *et al.*, 2005), but the two most widely discussed have been the ‘reproductive assurance’ hypothesis and the ‘automatic selection’ hypothesis. Under the former, selfing is favoured because it increases offspring production when outcross pollen availability or pollinator activity is sufficiently low to limit the number of seeds a plant can produce (Darwin, 1876; Stebbins, 1957; Baker, 1965). Under the latter, alleles that cause selfing have a 50 % transmission advantage over those that cause outcrossing because of their double representation in the seeds produced by self-fertilizing genotypes (Fisher, 1941). The reproductive assurance hypothesis has received empirical

support from a variety of recent studies, although several critical aspects of this hypothesis still need to be fully evaluated (Busch and Delph, 2012).

An alternative to the reproductive assurance hypothesis is the ‘time limitation’ hypothesis (Aarssen, 2000; Snell and Aarssen, 2005), which proposes that autogamous selfing has evolved most frequently in annuals because it results in a faster life cycle – and thereby increases seed production – in environments where seasonally deteriorating conditions limit the number of ovules that can be successfully matured into seeds, even in the absence of pollen limitation. Under such circumstances, selfing genotypes might be selectively favoured either directly (if selfing reduces the time interval between stigma receptivity and ovule fertilization) or indirectly (if selection for rapidly maturing plants or rapidly developing flowers results in the evolution of flowers with higher autogamous selfing rates due to reduced temporal (dichogamy) and/or spatial (herkogamy) separation of anthers and stigmas) (Guerrant, 1989; Aarssen, 2000; Runions and Geber, 2000; Armbruster *et al.*, 2002; Mazer *et al.*, 2004; Snell and Aarssen, 2005).

Currently, evidence in support of the time limitation hypothesis is largely indirect, and consistent with other interpretations. For example, some selfing taxa do flower earlier, show more rapid development of individual flowers and whole inflorescences, have shorter floral life spans and/or produce seeds more quickly than their outcrossing relatives (Vasek, 1977; Fenster *et al.*, 1995; Runions and Geber, 2000; Eckhart *et al.*, 2004; Mazer *et al.*, 2004; Snell and Aarssen, 2005; Dudley *et al.*, 2007; Wu *et al.*, 2010). However, this does not necessarily mean that selection for a faster life cycle caused the evolution of selfing, directly or indirectly: even if a faster life cycle was being favoured because of its advantages in a seasonally deteriorating environment, selfing could still have evolved for reasons of reproductive assurance. Similarly, the widely reported association between high selfing rates and small flowers (Goodwillie *et al.*, 2010) might be due to the selective advantage of small flowers under environmental conditions favouring faster life cycles, but has usually been interpreted as being due to the fitness benefits of reduced resource allocation to attractive structures in taxa that are not dependent on pollinators for seed production (Charlesworth and Charlesworth, 1987; Lloyd, 1987).

The time limitation hypothesis could apply to any environment where plants experience predictable, seasonally deteriorating conditions for seed production that are unrelated to pollinator activity. Such conditions could include drought, excessive heat or cold, low nutrient availability or extensive herbivory (Aronson *et al.*, 1992; Olsson and Ågren, 2002; Weintraub and Schimel, 2005; Franke *et al.*, 2006; Hall and Willis, 2006; Franks *et al.*, 2007; Sletvold *et al.*, 2015). The proposition that late season drought has favoured the evolution of higher selfing rates is a special case of the time limitation hypothesis and has been termed the ‘drought escape’ hypothesis by Dudley *et al.* (2015), although the idea itself can be traced back to the work of Moore and Lewis (1965), Vasek (1971) and Arroyo (1973). Indirect support for this hypothesis comes from studies showing that traits associated with drought escape (*sensu* Ludlow, 1989), including rapid growth, a faster life cycle, high photosynthetic and transpiration rates, and low water use efficiency (WUE), are more pronounced in selfing taxa than in their outcrossing relatives (Elle *et al.*, 2010; Mazer *et al.*, 2010; Wu *et al.*, 2010; Ivey and

Carr, 2012; Schneider and Mazer, 2016). Again, however, such associations do not mean that selection for a faster life cycle (and a drought escape physiology) caused the evolution of higher levels of autogamy. Better support for the hypothesis, at least in its indirect selection scenario, would be provided by evidence that traits promoting selfing evolve in response to selection for traits that promote drought escape. The most direct way of looking for such evidence is to perform an artificial selection experiment.

A related question is whether the life history and physiological traits associated with drought escape themselves evolve independently of one another or in a correlated fashion. Genetic correlations between early flowering and high gas exchange rates or low WUE have been found in several recent studies (Geber and Dawson, 1990, 1997; McKay *et al.*, 2003; Manzaneda *et al.*, 2015). Thus, selection for early flowering might cause the correlated evolution of high gas exchange rates and low WUE, and may even provide the genetic basis for the evolution of drought escape physiology if the correlations are due to pleiotropy (McKay *et al.*, 2003, 2008; Lovell *et al.*, 2013).

Here we describe the results of an artificial selection experiment designed to test the indirect selection scenario of the time limitation hypothesis in *Clarkia* (Onagraceae) – a particularly suitable genus for this purpose for three reasons. First, increased levels of autogamous selfing have arisen in multiple lineages owing to the evolution of reduced levels of dichogamy and herkogamy (Moore and Lewis, 1965; Vasek, 1977; Holtsford and Ellstrand, 1992; Eckhart and Geber, 1999; Mazer *et al.*, 2004; Moeller, 2006; Dudley *et al.*, 2007). Secondly, the flowering phenology of *Clarkia* populations makes them particularly vulnerable to the late spring droughts characteristic of much of their range in the western USA (Vasek and Sauer, 1971; Mazer *et al.*, 2010; Dudley *et al.*, 2015; Schneider and Mazer, 2016). Thirdly, studies of *Clarkia* sister taxa with contrasting mating systems have found that, when living sympatrically or at similar elevations, selfing taxa tend to have higher gas exchange rates (Mazer *et al.*, 2010) and to flower earlier and complete their life cycles sooner than their outcrossing relatives (Moore and Lewis, 1965; Vasek and Sauer, 1971; Vasek, 1977; Eckhart and Geber, 1999; Runquist *et al.*, 2014). This suggests that increased rates of selfing may have evolved in a correlated fashion with a faster life cycle and a drought escape physiology. Artificial selection experiments have two main advantages over more indirect approaches to addressing this question (Runions and Geber, 2000; Mazer *et al.*, 2004; Dudley *et al.*, 2007; Gould *et al.*, 2014; Schneider and Mazer, 2016). First, they are able to show explicitly whether populations are capable of evolutionary responses to both direct and indirect selection; this capability is only implicit in studies that estimate phenotypic correlations or those that estimate heritabilities and genetic correlations from common garden family-structured experiments. Secondly, they allow one to study the consequences of direct selection on single traits, rather than on multiple traits simultaneously, as is typically the case for field-based observational studies of natural selection (Campbell, 2009).

Using *Clarkia unguiculata* as our study species, we selected for early flowering or low herkogamy in two independent replicates from each of three southern California populations and investigated (1) whether early flowering lines also evolved reduced levels of herkogamy or dichogamy; (2) whether low herkogamy lines also evolved earlier flowering; and (3) whether

early flowering lines also evolved the higher photosynthetic and transpiration rates and the lower WUE characteristic of taxa with a drought escape physiology. The existence of correlated evolutionary responses in these traits would provide support for the indirect selection scenario of the time limitation hypothesis. The absence of such responses would suggest that the associations between mating system, life history and physiological traits observed in *Clarkia*, and possibly other taxa too, are due to the independent evolution of these traits.

MATERIALS AND METHODS

Overall design

There were four stages to the study: (1) seed collection from three California populations of *C. unguiculata*; (2) creation of a set of greenhouse-raised baseline populations to minimize environmentally based maternal effects prior to the start of selection; (3) three generations of selection in the greenhouse; and (4) planting the offspring of the third generation of selection into the field in California and comparing the morphological, life history and physiological traits of control vs. selected lines. Stage 4 was a necessary part of the study because trait expression under greenhouse conditions might be very different from that in the natural habitat during a natural seasonal life cycle. Most of the greenhouse phase of the study was conducted at the University of St. Thomas, St. Paul, MN (UST), although for logistical reasons the first generation of selection in one of six replicates and the third generation of selection in two of six replicates took place at the University of California, Santa Barbara (UCSB).

Study system

Clarkia unguiculata is a primarily outcrossing, protandrous, diploid species endemic to the California floristic province. Although it is self-compatible, high outcrossing rates (Vasek, 1965; Ivey *et al.*, 2016; Hove *et al.*, 2016) are maintained through both dichogamy and herkogamy (Vasek, 1971, 1977; Dudley *et al.*, 2007; Schneider and Mazer, 2016). Populations of *C. unguiculata* occupy open oak woodlands and road cuts in the foothills and mountains surrounding California's San Joaquin Valley, with several disjunct populations occurring in San Diego County and the Baja Peninsula (Jonas and Geber, 1999). For our study, seeds from 320 maternal plants were collected from each of three wild populations of *C. unguiculata* in Kern County, California in July–August 2007. Locations and elevations of these sites were as follows: Granite Road (G): 35°41.45'N, 118°43.91'W, 869 m; Jack and Stage (J): 35°47.74'N, 118°42.15'W, 1006 m; and Mill Creek (M): 35°32.24'N, 118°36.84'W, 774 m. Seed families were stored in individual coin envelopes inside sealed plastic bags containing silica gel desiccant, and refrigerated until use.

Seed germination and plant cultivation

For each generation, 30–40 seeds from each maternal plant were sprinkled onto the surface of an agar-filled Petri dish,

refrigerated in the dark for 7 d then allowed to germinate at room temperature in the greenhouse or in an illuminated growth chamber (14:10 h light:dark). When the cotyledons had expanded and roots were 5–15 mm long, three seedlings were transplanted into each of six Ray Leach 'Cone-tainer' cells (Stuewe & Sons, Inc., Tangent, OR, USA), filled with soil-less potting mix (four parts Pro-Mix® BX Biofungicide™, two parts perlite, two parts vermiculite, one part sand), to which was added four fertilizer beads (Osmocote® 19-6-12) sieved to achieve a uniform diameter of 2–4 mm. Seedlings were misted daily until established, and thinned to two per cell after 7 d. Spacing between cells in their racks was increased regularly as plants grew and, once they had reached 30 cm in height, one plant per cell was attached to a wire stake taped to the outside of the cell and the second plant was cut off at the base of the stem. Racks were moved randomly to new locations within their particular greenhouse bench every 2 d to minimize the effects of small-scale environmental influences within the greenhouse. Environmental differences among benches were minimal, given their orientation relative to potential sources of variation in light and temperature. Plants were watered from below as needed prior to staking and then from above every 2 d after staking. Greenhouse lights (Sun System III® (Sunlight Supply, Inc., Vancouver, WA, USA) fitted with 400 W Hortilux® Super HPS lamps (Eye Lighting International, Mentor, OH, USA) were used to provide a 12:12 h light:dark cycle between the autumn and spring equinoxes. Between the spring and autumn equinoxes, plants received a natural daylight cycle, using a combination of sunlight and greenhouse lights.

Generation of the baseline population

Between October 2007 and April 2008, the 320 maternal seed families within each population were randomly assigned to 160 pairs, and two offspring per family were raised to maturity. In a small fraction of cases, pair members were reassigned to ensure that their flowering periods overlapped. Each plant was reciprocally cross-pollinated by hand with pollen from one offspring from its pair member, and each pair was assigned to one of two independent replicates (A and B). Recipient flowers were emasculated prior to stigma receptivity to prevent self-pollination. These pollinations therefore generated 320 maternal seed families in each replicate. Within each replicate, one maternal family was randomly chosen from each of the 160 pairs to create a baseline population of 160 maternal families per replicate (Fig. 1A).

First generation of selection

Between June 2008 and April 2010, six offspring from each of the 160 maternal families in each of the two replicate lines were raised to maturity. A small number of families (2–8) were lost from each replicate because seeds failed to germinate or because seedlings died. Thirty families from the 160 in each replicate were randomly selected to create a control outcrossed line and, from the remaining 130 families, the 30 most rapidly maturing families were selected to create an early flowering outcrossed line, based on the mean number of days between seedling

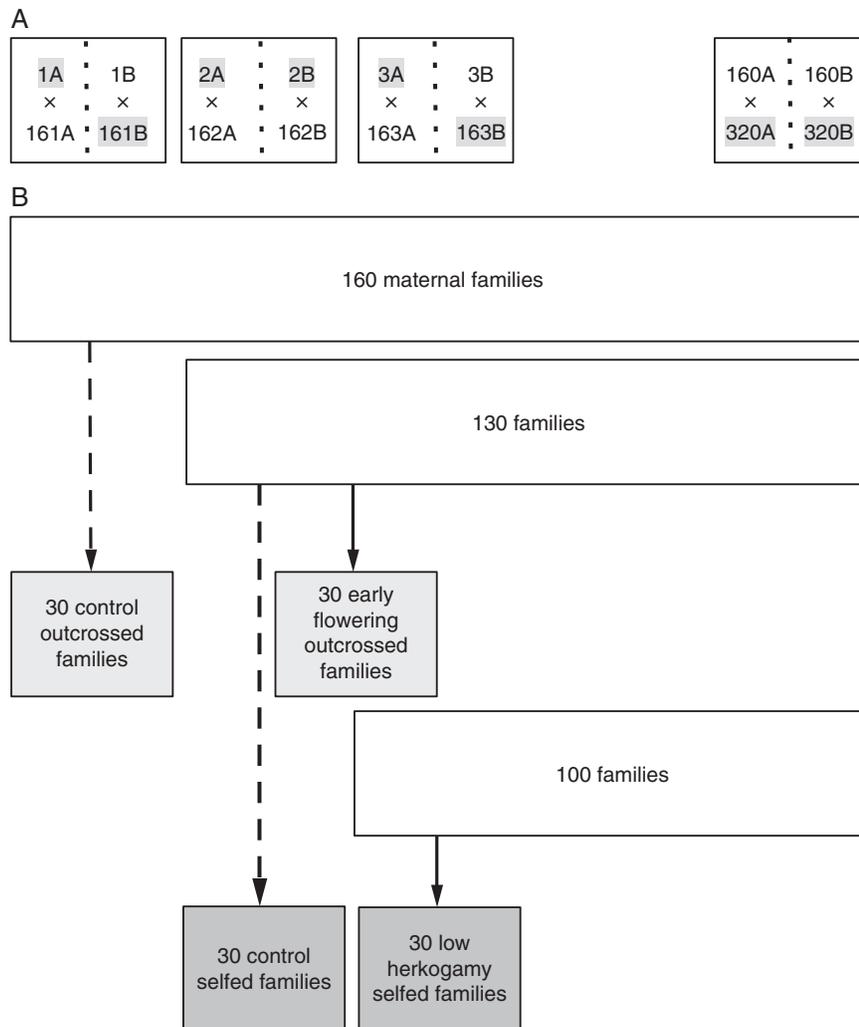


FIG. 1. (A) Experimental design for creation of the baseline generation. Numbers indicate seed families from field collections: plants sharing a number are maternal half-sibs assuming different pollen parents. Letters refer to the two independent replicates created from each field population. × = reciprocal cross-pollination following emasculation of recipient flowers. Grey boxes indicate which of the two potential maternal plants within each cross was chosen at random to produce seed for the first generation of selection. Pairs are numbered sequentially for clarity of presentation only: in reality, pair membership was assigned at random (see the Materials and Methods for details). (B) Experimental design for the first generation of selection. Dashed arrows indicate control plants that were chosen randomly from the set above. Solid arrows indicate selected plants chosen from the set above using the criteria described in the Materials and Methods. Families in the early flowering outcrossed group could also potentially be represented in the control selfed or low herkogamy selected group, although different individual plants were used in the different lines. In subsequent generations of selection, families were chosen randomly (controls) or selected from within each of the four groups. See the Materials and Methods for details.

transplant and the opening of the first flower on the first three plants within the family to start flowering. From the same 130 families, 30 were randomly selected to create a control selfed line, and from the remaining 100 families, 30 were selected to create a low herkogamy selfed line (Fig. 1B). Because of this design, some families were in both the early flowering outcrossed line and either the control selfed line (0–11 families per replicate) or the low herkogamy selfed line (5–8 families per replicate), at least for the first generation of selection. For the low herkogamy selfed lines, selected families were chosen on the basis of the mean herkogamy scores of three randomly chosen plants within each family, based on examination of all the open flowers on a plant that had receptive stigmas on one day in the middle of its blooming period. Herkogamy scores were: 1 = at least one flower with a receptive stigma showing anther–stigma

contact; 2 = no flowers showing anther–stigma contact but at least one with an anther–stigma separation ≤ 3 mm; 3 = no flowers showing an anther–stigma separation ≤ 3 mm.

In the early flowering and control outcrossed lines, each of the 30 maternal families was randomly assigned for pollination of one of the other 29 families, without any reciprocal pollination. Three plants from the set of six within each family were randomly chosen for this purpose. Recipient flowers were emasculated prior to stigma receptivity to prevent self-pollination and subsequently hand pollinated. In the low herkogamy and control selfed lines, flowers from three randomly chosen plants within each family were self-pollinated by hand. For all lines, fruits were collected as they started to dehisce and seeds from all three recipient plants within each family were mixed prior to the start of the next generation.

Second and third generations of selection

For these generations, the basic protocol was the same as for the first generation, with the following differences. First, seven plants were raised from each maternal family to increase the number of available individuals if survival to maturity was low. Secondly, a more quantitative criterion was used as the basis for selection on herkogamy. Each plant was examined on at least two occasions separated by 4–5 d, the herkogamy status of all flowers with receptive stigmas was examined and the mean value for that date recorded. The herkogamy status of the whole plant was estimated as the average of these mean values. The herkogamy status of individual flowers was categorized as 1 = anther–stigma distance 0–1 mm; 2 = anther–stigma distance 1–3 mm; 3 = minimum anther–stigma distance >3 mm.

For the second generation of selection, 15 of the 30 families in each line were selected for the targeted trait; for the third generation, six families were selected from those 15. Control families were chosen randomly; selected families were chosen on the same basis as for the first generation. As with the first generation, within each outcrossed line each maternal family was randomly assigned to pollinate one of the other families without reciprocal pollination. Within each of these families, four plants were randomly chosen for pollination and seed production; the remaining three were used for the measurement of herkogamy and autogamous fruit production. For the selfed lines, herkogamy status was recorded for all seven plants and, in the chosen families (both control and selected lines), four of the seven plants were randomly assigned to be self-pollinated. The remaining three plants were used for measurement of autogamous fruit production, which was recorded as the number of fruits produced by the first ten flowers to open on the main stem of the plant. For the two replicates of the M population, plants were small, so all seven were self-pollinated to ensure sufficient seed production. Thus, autogamous fruit production was not measured in these replicates. Selection differentials (calculated as the difference between the mean of the selected family means and the mean of all family means in the pre-selection population) for each generation of selection are provided in Supplementary Data Table S1.

Field cultivation and measurements

For this stage of the study, only the early flowering and their respective control lines were used. After three generations of selection, the seeds from each of the six remaining maternal families in each line were divided into 60 sets of ten and temporarily stored in individual microcentrifuge tubes. From 19–23 January 2013, seeds from all 70 useable maternal families (3 populations × 2 replicates × 2 selection lines × 6 families = 72 families; two families did not produce enough seeds) were sown in an experimental garden created at a field site located in an open oak woodland where both *C. unguiculata* and *C. exilis* had been observed in previous years. This site, Upper Richbar (Kern River Canyon, Kern County, CA, USA: 35°28.46'N, 118°43.12'W, 436 m), consisted of a level meadow on pliable loam located between a boulder-strewn field and the base of a steep slope. We created sixty 40 cm × 120 cm plots located approx. 1–3 m apart (depending on microtopography

and the presence of large rocks in the soil). Each plot consisted of a grid of 5 rows × 15 columns, with the centres of the cells of the grid being 10 cm apart. Within each plot, the ten seeds in each microcentrifuge tube from all 70 maternal families were sown in a small depression in one of the 75 cells, assigned to maternal families at random. The remaining five cells in each were left empty. A total of 42 000 seeds were sown (60 plots × 70 maternal families × 10 seeds/family).

Because of the lack of precipitation, it was necessary to water the plots to induce germination and to support the germinating seedlings, juveniles and adults throughout the winter and spring of 2013. Two or three times a week until the end of the experiment on 7 July, each plot was mist-watered to achieve the equivalent of 3.5 mm of rainfall per watering, approximately reflecting natural seasonal rainfall patterns in the region (Schneider and Mazer, 2016). From 13 to 21 March, the seedlings in each plot were thinned to one healthy plant per cell. As plants matured, the date of opening of the first flower was recorded for each flowering individual ($n = 1965$). For 4–5 plants per maternal family ($n = 304$ individuals), we recorded the node number of the first flower (node 1 being the first node above the cotyledons), the first day of anther dehiscence and the first day of stigma receptivity of the first, third and fifth flower on the main stem, and the anther–stigma distance at the time of stigma receptivity of one representative flower (ranging from the first to the 12th flower produced by the main stem; $n = 223$ individuals). There was no significant difference between control and early flowering lines in the node of the flower used to measure anther–stigma distance (Kruskal–Wallis test: $Z = -1.231$, $P = 0.219$, $n = 223$). There was also no significant correlation between flower node and anther–stigma distance for measured flowers (Pearson's $r = -0.071$, $P = 0.289$, $n = 223$). Thus, comparisons of anther–stigma distance between control and early flowering lines were not biased by the node of the measured flower.

Daytime gas exchange rates (photosynthesis, A and transpiration, E) and instantaneous WUE ($A/E \times 10^{-4}$) were measured using an LI-6400 portable photosynthesis system and leaf chamber fluorometer (LI-COR, Inc., Lincoln, NE, USA). Measurements were made both Early (9–14 April) and Late (8–14 May) in the season, on 2–4 plants per family. The two measurement periods were timed to coincide with plants being in early bud (Early) or in the middle of flowering (Late), and to reflect increasing seasonal drought stress for *Clarkia* (Vasek and Sauer, 1971; Mazer *et al.*, 2010; Dudley *et al.*, 2015; Schneider and Mazer, 2016). If selection for early flowering led to the evolution of a drought escape physiology, selected lines should have had higher gas exchange rates than their respective controls. However, we had no *a priori* expectation as to whether these higher rates would be observed under all environmental conditions, only under low stress (Early) or only under high stress (Late). On each day, measurements were made between 08.00 h and 15.30 h (mostly between 09.00 h and 13.00 h), starting at least 30 min after plants were exposed to direct sunlight and ending before plants showed signs of mid-day stomatal closure or wilting. Basic LI-6400 settings were as follows: photosynthetically active radiation (PAR) = 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$; flow = 500 $\mu\text{mol s}^{-1}$; reference $[\text{CO}_2] = 400 \mu\text{mol mol}^{-1}$. Stability criteria were: photosynthesis = slope <1 for 10 s; conductance = slope <0.05 for 10 s. Leaf samples were taken

from one plant per family during both Early (15 April) and Late (11 May) measurement periods for subsequent analysis of the carbon isotope ratio ($\delta^{13}\text{C}$ ‰), which can be used as an indicator of long-term integrated WUE (Dawson *et al.*, 2002). Analysis of these samples was carried out at the Cornell Stable Isotope Laboratory (<http://www.cobsil.com>). Further details of LI-6400 settings and gas exchange measurement methods are provided in Dudley *et al.* (2015).

Statistical analyses of greenhouse data

We studied the effects of our artificial selection regime by comparing the mean trait value of each selected line with that of its respective control after two generations of selection. We used one-tailed tests for direct responses to selection (because we had an *a priori* expectation for the direction of response) and two-tailed tests for correlated responses (because we had no *a priori* expectation for the direction of any genetic correlations).

Days to first flower and herkogamy scores were both normally distributed for both lines of all replicates except for herkogamy scores in the two replicates of the M population. Thus, we used Kruskal–Wallis tests for the latter two comparisons and *t*-tests for all others. The proportion of flowers producing fruits by autogamous selfing was arcsine square-root transformed before analysis, but the data were still significantly non-normal in some replicates because many plants produced no fruits. Thus, Kruskal–Wallis tests were used to compare autogamous fruit production in all replicates.

We report raw *P*-values because the control vs. selected contrasts were all planned, but, as an additional conservative test, we also adjusted alpha levels to control for multiple comparisons using the false discovery rate (FDR) criterion (Benjamini and Hochberg, 1995; García, 2004) and indicate which results remain significant after these adjustments. All statistical analyses were performed using JMP[®] Pro 12.0 (SAS Institute Inc., Cary, NC, USA).

Statistical analyses of field data

We studied the effects of our artificial selection regime under field conditions after three generations of selection. Preliminary analysis of the data revealed that for most of the response variables there was statistically significant variation among plots. For gas exchange rates and WUE, there were also statistically significant effects of air temperature, relative humidity and measurement date. (Temperature and humidity were measured instantaneously with the LI-6400 at the same time as each gas exchange rate measurement.) To control for these effects, and to allow data to be pooled across plots, we first generated residual values for individual plant measurements and then calculated the maternal family means of these residuals. For days to first flower, anther–stigma distance, node of first flower and $\delta^{13}\text{C}$, residuals were calculated as the deviation of each individual's phenotype from its plot mean. Anther–stigma distance and node of the first flower did not differ significantly among plots, but analysis of variance (ANOVA) *R*² values were similar to those for the date of the first flower (0.20–0.22), so residuals were used for consistency of analysis. Protandry was calculated

as the number of days between first anther dehiscence and first stigma receptivity for each of the three flowers examined, and a mean value calculated for each plant. Preliminary analysis showed that family mean protandry values were positively correlated with family mean days to first flower (control families, Pearson's $r = 0.624$, $P < 0.001$, $n = 34$; selected families, $r = 0.253$, $P = 0.138$, $n = 36$; all families, $r = 0.477$, $P < 0.001$, $n = 70$). Thus, individual plant residuals were estimated from a model including both plot and days to first flower.

For gas exchange rates and WUE, we examined several preliminary models for residual calculation because air temperature, relative humidity and measurement date were correlated with one another. We then chose the simplest model that explained most of the variation in *A*, since this variable was expected to be most strongly influenced by selection on days to first flower if rapid maturation was mediated by higher photosynthetic rates. For Early measurements, the chosen model included plot and air temperature as independent variables; for Late measurements, the chosen model included plot and measurement date. Using more complicated models had no influence on our final analyses because the residuals generated from these models were highly correlated with the residuals generated from our chosen models.

Variation in the family mean residuals generated from all the above models was analysed using a series of nested ANOVAs, with population (G, J, M), line (control vs. selected) and the population \times line interaction treated as fixed effects, and replicate (A vs. B, nested within population) and replicate \times line (also nested within population) treated as random effects. If the overall line effect was significant, control vs. selected lines within each replicate were compared using a *t*-test on least squares means – one-tailed for the effects of direct selection on days to first flower and two-tailed for the effects of correlated evolution on node of the first flower, anther–stigma distance and protandry. As with the greenhouse analyses, we report both raw *P*-values and values adjusted for multiple comparisons within each ANOVA.

RESULTS

Greenhouse analyses: responses to direct selection on days to first flower and herkogamy

Days to first flower and herkogamy score both responded to direct selection. After two generations of selection, greenhouse-raised plants in all six replicates of the early flowering lines started to flower significantly earlier than their respective controls (Fig. 2A) with mean differences ranging from 1.46 d (3.79 %) to 6.42 d (9.49 %) (Supplementary Data Table S1). Similarly, five of the six replicates selected for reduced herkogamy had significantly lower herkogamy scores than their respective controls, with the difference in the sixth replicate being marginally significant (Fig. 2B). Mean differences ranged from 0.22 (7.68 %) to 0.54 (20.46 %) (Supplementary Data Table S1). Differences in mean days to first flower among populations partly reflected developmental responses to seasonal photoperiod and temperature variation in the greenhouse: plants germinated in autumn and grown in winter took longer to flower than those germinated in winter and grown in

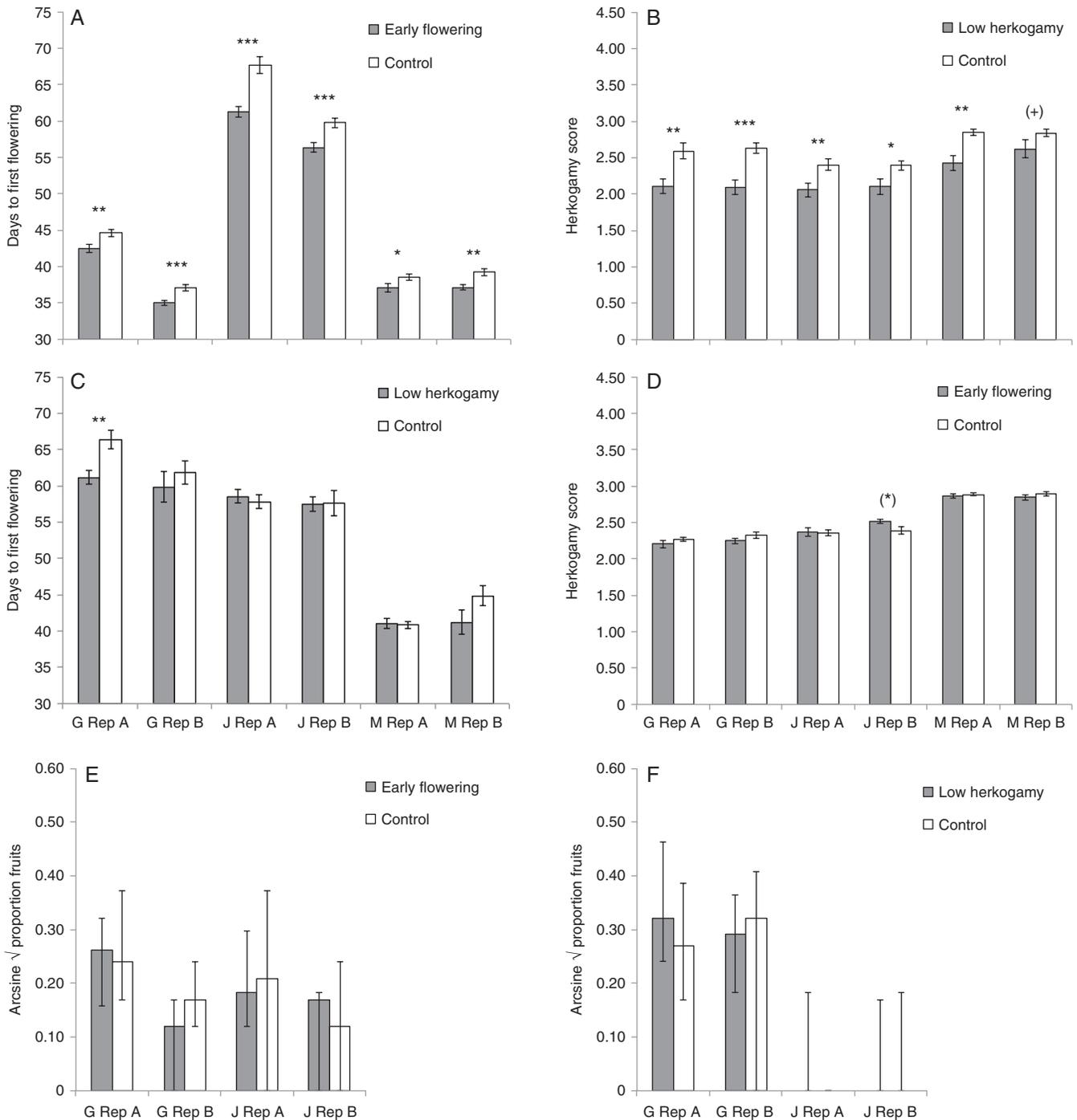


FIG. 2. Days to first flowering, herkogamy scores and autogamous fruit set of greenhouse-raised plants after two generations of selection. (A) Direct selection on early flowering. (B) Direct selection on low herkogamy. (C) Indirect selection on early flowering due to direct selection on low herkogamy. (D) Indirect selection on low herkogamy due to direct selection on early flowering. (E) Autogamous fruit set of early flowering lines and their controls. (F) Autogamous fruit set of low herkogamy lines and their controls (not measured for the M population). For (A–D), bars show the mean \pm 1 s.e.; for (E) and (F) they show medians and interquartile ranges. In (F), medians for all lines of the J population were zero; the interquartile range for the control line of replicate A was also zero. $n = 15$ families for all groups. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, + $P < 0.1$; one-tailed tests for direct selection comparisons; two-tailed tests for indirect selection comparisons (t -tests for A–D; Kruskal–Wallis tests for E and F). All significant differences remained significant at $P < 0.05$ or lower after adjustment for multiple comparisons using the false discovery rate (FDR) criterion (Benjamini and Hochberg, 1995; García, 2004), except for those in parentheses.

spring. However, there was also a population effect on days to first flower under common garden conditions in the field (see below), indicating a genetic, as well as an environmental, component to this variation.

Greenhouse analyses: correlated evolutionary changes

Despite the clear responses to direct selection, there was little or no evidence that selection for early flowering led to correlated

evolutionary changes in herkogamy or vice versa. Four of the six low herkogamy lines flowered earlier than their respective controls, but only one of these responses was statistically significant (Fig. 2C). Similarly, although four of the six early flowering lines had lower mean herkogamy scores than their respective controls, none of these differences was statistically significant. In fact, in one of the other two replicates, the early flowering line had a marginally significant higher herkogamy score than its control ($P = 0.048$; Fig. 2D). Moreover, when the 15 families in each selected line were pooled with their 15 respective controls, only one of the 12 data sets showed a significant correlation among family means between days to first flower and herkogamy score (Table 1).

Autogamous fruit set per plant was generally very low (overall mean proportion per ten flowers = 0.05; range = 0–0.9; $n = 1130$ plants, 828 of which produced no fruits) and also quite variable both among families (mean = 0.051; range = 0–0.5; $n = 224$ families, 177 of which produced no fruits) and among plants within a family. There were no significant differences in fruit set between control and selected lines, regardless of whether selection was for early flowering (Fig. 2E) or for low herkogamy (Fig. 2F).

Field analyses: direct selection on days to first flower and correlated evolutionary changes in floral traits

As in the greenhouse, lines selected for early flowering started to flower significantly earlier than control lines in the field (Table 2), and this effect was consistent and statistically significant across all six replicates (mean difference = 1.10 d; range = 0.75–1.53; Fig. 3A). Early flowering lines also tended to begin flowering on lower nodes, although this effect was only marginally significant in the ANOVA (Table 2), was not consistent across populations, and was only marginally significant for two of the six replicates (Fig. 3B). Early flowering lines did not differ significantly from controls in anther–stigma distance or protandry (Fig. 3C, D; Table 2). Population of origin affected days to first flower and the node of the first flower, but not anther–stigma distance or protandry (Table 2). Raw data for floral traits are provided in (Supplementary Data Fig. S1; Table S2).

TABLE 1. Pearson's correlation coefficients among maternal family means between mean herkogamy score and mean days to first flower for greenhouse-raised plants after two generations of selection

Population	Replicate	Lines selected for early flowering		Lines selected for low herkogamy	
		<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
J	A	0.094	0.619	0.084	0.658
J	B	–0.148	0.436	0.037	0.848
G	A	0.010	0.958	0.420	0.021
G	B	0.118	0.662	0.043	0.821
M	A	0.022	0.909	–0.174	0.357
M	B	–0.278	0.135	0.049	0.815

$n = 30$ families (control + selected) in each of the six population \times replicate combinations.

Field analyses: correlated evolutionary changes in gas exchange rates, WUE and $\delta^{13}C$

There were no significant effects of population, line or their interaction on photosynthetic rate *A*, transpiration rate *E*, WUE or $\delta^{13}C$, regardless of whether these measurements were made Early, when plants were in bud and under relatively low drought stress, or Late, when plants were in flower and under relatively high drought stress (Fig. 4; Table 2). Raw data for gas exchange traits are provided in Supplementary Data Fig. S2 and Table S3.

DISCUSSION

The purpose of this study was to evaluate the indirect selection scenario of the time limitation hypothesis in a genus of plants where autogamous selfing has repeatedly evolved in taxa with largely outcrossing ancestors. Specifically, our goals were to assess whether selection for rapid maturation (early flowering) might cause the correlated evolution of (1) reduced levels of herkogamy or dichogamy, and thus higher rates of autogamous selfing; and (2) higher photosynthetic and transpiration rates and reduced WUE, characteristic of species with a drought escape physiology. Our results suggest that this scenario does not explain the evolution of autogamous selfing in *Clarkia*.

The evolution of life history and mating system

Days to first flower and anther–stigma distance both responded to direct selection. After just two generations of selection, days to first flower was significantly lower in selected lines than in controls in all six replicates; anther–stigma distance was significantly shorter in selected lines in five of the six replicates. After three generations of selection, days to first flower was also significantly lower in selected lines in all six replicates even under field conditions, although mean differences were smaller than in the greenhouse, presumably because of the greater environmental heterogeneity in the field or genotype \times environment interactions. Our experimental design precluded quantification of responses to selection (*R*) and thus heritabilities (h^2). However, our results are broadly similar to a variety of recent studies that have estimated the heritabilities of floral traits (Ashman and Majetic, 2006) including those that have demonstrated significant evolutionary responses to artificial selection on such traits, as well as on the date of first flowering (Worley and Barrett, 2000; Lendvai and Levin, 2003; Delph et al., 2004a; Burgess et al., 2007; Van Dijk, 2009; Conner et al., 2011).

Despite these responses to direct selection, there was no evidence that selection on flowering date caused the correlated evolution of reduced levels of herkogamy or dichogamy. There was also little evidence that selection for shorter anther–stigma distances caused the correlated evolution of more rapid maturation and earlier flowering, but data on this question are only available for the greenhouse stage of the experiment because low herkogamy lines and their controls were not planted in the field.

TABLE 2. Summary of nested ANOVAs for field-raised plants after three generations of selection for early flowering

	<i>n</i> (families)	Model <i>R</i> ²	Fixed effects <i>F</i> ratio (<i>P</i> -value)		
			Population (d.f. = 2)	Line (d.f. = 1)	Population × Line (d.f. = 2)
Days to first flower	70	0.88	84.386**	21.457*	0.087
Node of first flower	70	0.46	12.084*	6.757+	5.372+
Anther–stigma distance	69	0.26	0.841	1.043	1.779
Protandry	70	0.22	0.328	0.019	0.597
Photosynthesis <i>A</i> (Early)	70	0.09	0.758	0.194	2.704
Transpiration <i>E</i> (Early)	70	0.16	1.405	1.357	1.484
WUE (Early)	70	0.08	1.273	0.726	0.392
δ ¹³ C (Early)	68	0.11	0.229	0.0007	2.506
Photosynthesis <i>A</i> (Late)	64	0.17	0.669	0.008	1.144
Transpiration <i>E</i> (Late)	64	0.10	0.692	0.011	0.923
WUE (Late)	64	0.00	1.647	0.906	0.626
δ ¹³ C (Late)	49	0.31	4.396	0.604	1.686

Population refers to the locations of the three wild populations (G, J and M) from which seeds were originally collected. Line refers to control or early flowering selected lines.

Random effects in the model (replicate nested within population and replicate × line nested within population) are not shown: the 95 % confidence intervals for their variance components all enclosed zero. See the Materials and Methods for details.

***P* < 0.01; **P* < 0.05; +*P* < 0.1.

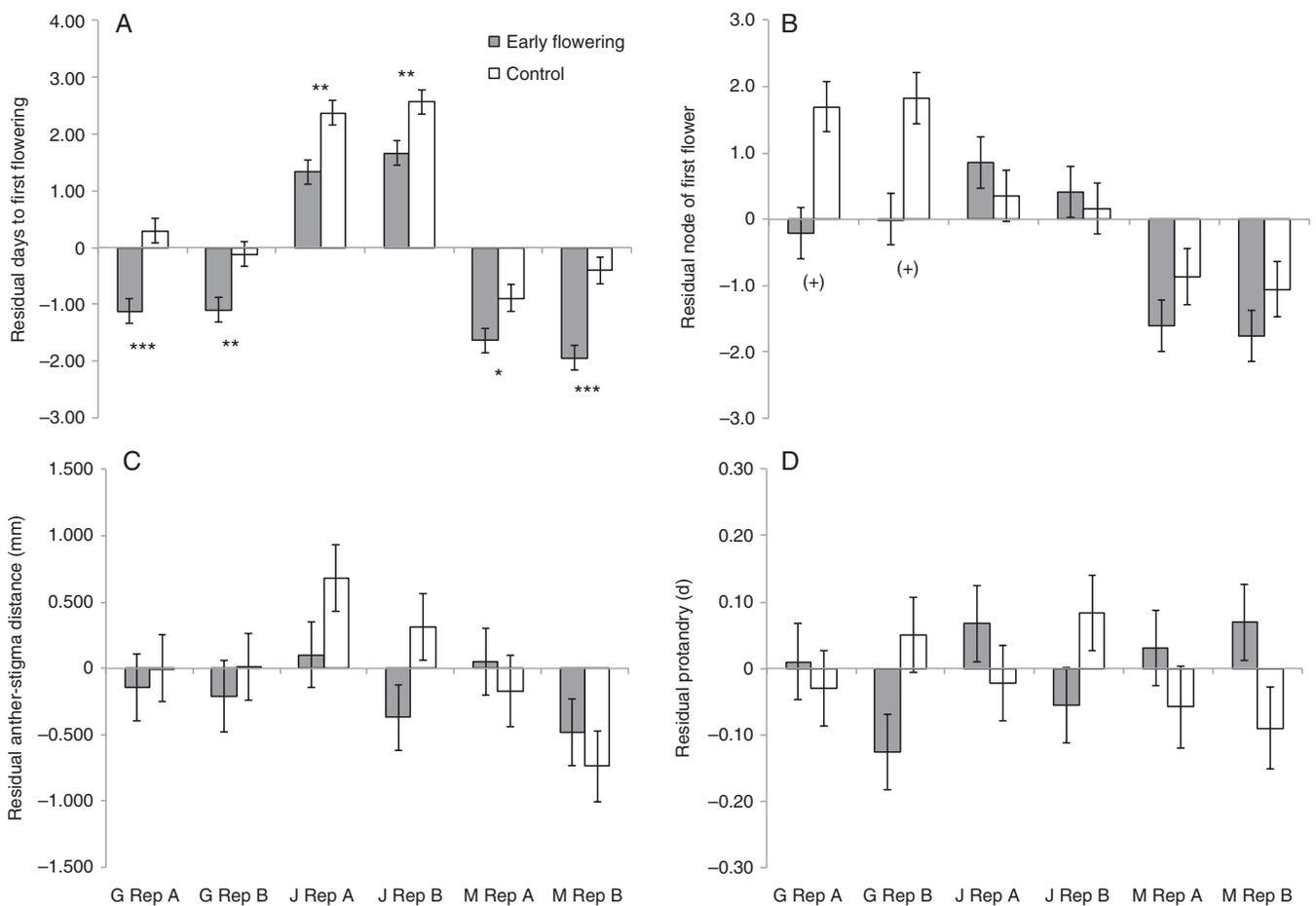


FIG. 3. Days to first flower, node of first flower, anther–stigma distance and protandry of field-raised plants after three generations of selection for early flowering. Bars show the least squares means \pm 1 s.e. of residual values from plot means (A–C) or from a model incorporating plot mean and days to first flower (D). *n* = 6 families for all groups except M Rep A and Rep B control lines, for which *n* = 5 families. See the Materials and Methods for details. Raw data are provided in Supplementary Data Fig. S1 and Table S2. ****P* < 0.001, ***P* < 0.01, **P* < 0.05, +*P* < 0.1; one-tailed *t*-tests for direct selection comparisons (days to first flower); two-tailed tests for indirect selection comparisons (node of first flower, anther–stigma distance, protandry). All significant differences remained significant at *P* < 0.05 or lower after adjustment for multiple comparisons using the false discovery rate (FDR) criterion (Benjamini and Hochberg, 1995; García, 2004), except for those in parentheses.

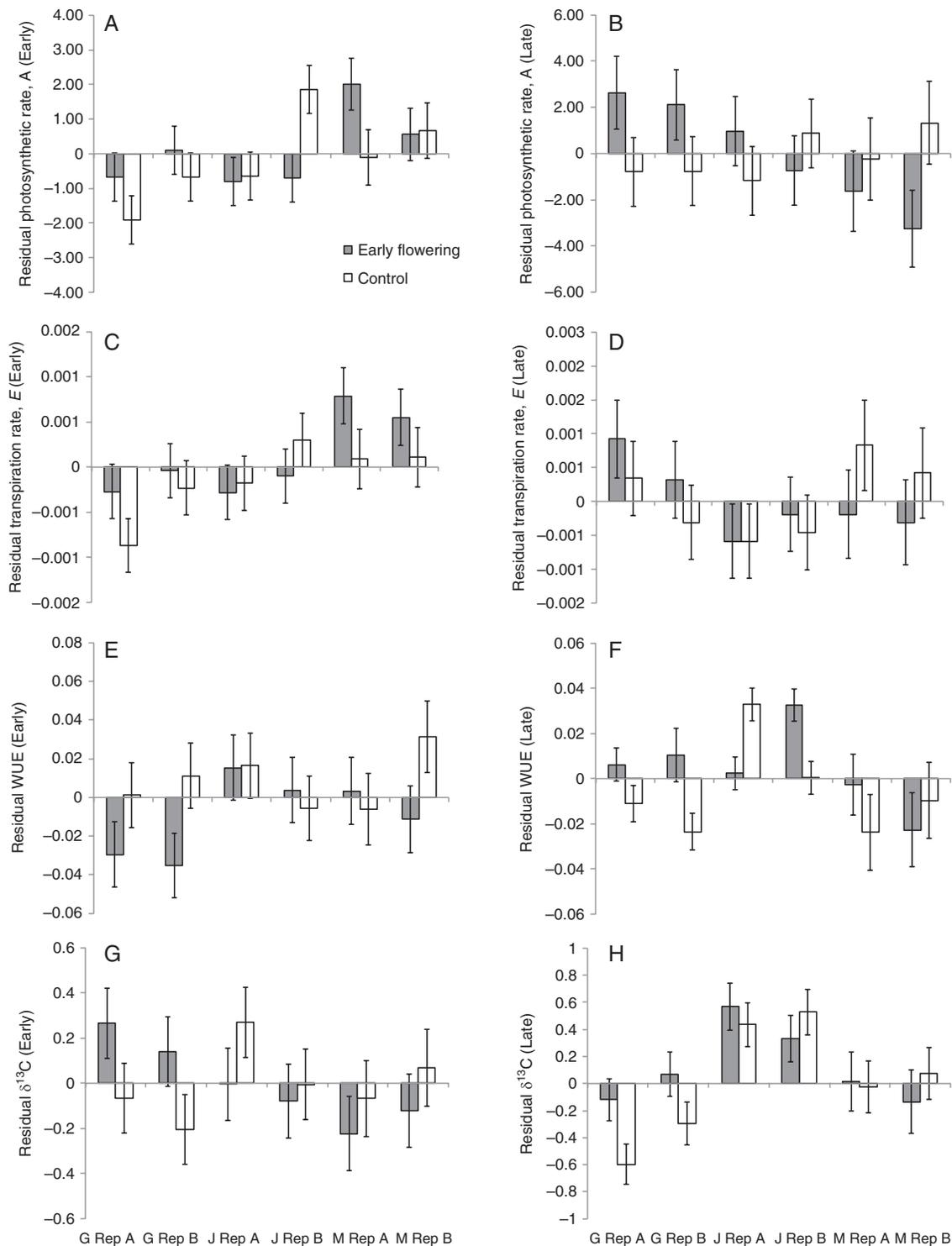


FIG. 4. Gas exchange rates and water use efficiencies (WUEs) of field-raised plants after three generations of selection for early flowering. Early season measures are shown on the left-hand side, late season measures on the right. $\delta^{13}\text{C}$ is a measure of integrated WUE over the lifetime of sampled leaves (see the Materials and Methods for details). Units of measurement are: A ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$); E ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$); WUE ($\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O} \times 10^{-4}$); $\delta^{13}\text{C}$ (‰). Bars show the least squares means \pm 1 s.e. of residual values after controlling for variation among plots and (for gas exchange rates and WUE) air temperature or measurement date. See the Materials and Methods for details. Raw values are provided in Supplementary Data Fig. S2 and Table S3. For gas exchange rates and WUE, $n = 6$ families for all groups except the M Rep A and Rep B control lines, for which $n = 5$ families. For $\delta^{13}\text{C}$, $n = 4$ –6 families for all groups except the M Rep A early flowering line, for which $n = 1$ family.

Although no previous studies of *Clarkia* have directly evaluated the potential for correlated evolutionary changes in floral

and life history traits, several have indirectly assessed this potential by estimating trait correlations among families or

among populations. Some of these studies have found little evidence of such correlations, either among floral traits (Runions and Geber, 2000; Gould *et al.*, 2014) or among floral and life history traits, including days to first flower vs. protandry (Mazer *et al.*, 2004) and days to first flower vs. herkogamy (Gould *et al.*, 2014). In contrast, Dudley *et al.* (2007) found consistent among-family floral trait correlations in all four taxa studied but (with the exception of *C. xantiana* ssp. *parviflora*) inconsistent and mostly non-significant correlations between floral and life history traits. Schneider and Mazer (2016) found significant among-family correlations between floral and life history traits in both *C. exilis* and *C. unguiculata*, including statistically significant correlations between herkogamy and days to first flower in both species (positive in *C. unguiculata*; negative in *C. exilis*). However, for *C. unguiculata*, this correlation was no longer significant after statistically controlling for seed size and population of origin.

In summary, several recent studies of *Clarkia*, including the work presented here, provide little support for the hypothesis that the repeated evolution of autogamous selfing in this genus has been caused by indirect selection due to genetic correlations between floral traits that influence the selfing rate (dichogamy and herkogamy) and traits that influence the whole-plant maturation rate (days to first flower). More broadly, our results can be compared with a variety of recent studies showing that, although indirect selection due to genetic correlations may cause evolutionary change in many floral, inflorescence and phenological traits [Worley and Barrett, 2000; Lendvai and Levin, 2003; Delph *et al.*, 2004a; Burgess *et al.* 2007; Van Dijk, 2009; Galloway and Burgess, 2012, but see Delph *et al.* (2004b) and some results of Worley and Barrett (2000) for counterexamples], such correlations tend to be stronger within classes of traits than between such classes. For example, Ashman and Majetic (2006) found stronger positive correlations within corolla traits, within primary sexual traits, and within inflorescence traits than between corolla and inflorescence traits or between floral and vegetative traits. Our finding that selection on a phenological trait (days to first flower) did not cause the correlated evolution of floral traits (herkogamy and dichogamy) – or vice versa for herkogamy and days to first flower – is consistent with the pattern found by Ashman and Majetic (2006).

One caveat to our conclusions is that artificial selection was imposed for only 2–3 generations. We cannot completely discount the possibility that weak genetic correlations exist and that these might manifest themselves after many more generations of selection. However, if that were true, we might have expected any correlated evolutionary changes to be in a consistent direction, even if they were not large or statistically significant. This was not the case, either among populations or across generations within a population (Figs 2 and 3). It is also worth pointing out that selection strengths in our study were relatively large. For days to first flower, mean standardized selection differentials (S') were -1.10 , -0.65 and -0.74 in the first, second and third generations of selection, respectively; for herkogamy they were -1.23 , -0.78 and -0.85 , respectively. In contrast, Geber and Griffen's (2003) review of natural selection in plant populations found that S' had a mean value of approx. 0.18 for phenological and developmental traits, and several recent studies of floral traits have estimated S' to range from 0.02 to 1.04, with a mean across all estimates of 0.43 (Campbell, 1996;

O'Neil, 1997; Fishman and Willis, 2008; Bartkowska and Johnston, 2012). Thus, selection strengths imposed during our experiment were not unrealistic, but were on the upper bounds of those reported from non-experimental, field-based studies. Our failure to detect indirect responses to these strengths of direct selection suggests that, if genetic correlations are present, they are likely to be weak. Similar considerations apply to the possibility that indirect responses occurred but were masked by genetic drift, given the relatively small number of families per line. If drift were sufficient to overwhelm any indirect responses, such responses would probably be too weak to have important consequences for trait evolution in natural populations. Moreover, the potential effects of drift were mitigated in our experiment by the family selection design, with 3–4 plants contributing to the next generation through both the maternal and paternal sides of each family.

In addition to the caveat discussed above, it is also worth emphasizing that our conclusions are restricted to the indirect selection scenario of the time limitation hypothesis. Higher selfing rates might also be favoured directly, even if plants were not pollen limited, if selfing led to earlier ovule fertilization than outcrossing (Aarssen, 2000). In principle, this could be studied experimentally by comparing the relative fitness of plants receiving hand pollinations of self-pollen immediately upon stigma receptivity (simulating prior selfing) with that of open-pollinated controls and with that of plants receiving hand pollinations of self-pollen towards the end of stigma receptivity (simulating delayed selfing, to determine whether plants were pollen limited). However, we know of no studies in which such experiments have yet been carried out.

The evolution of life history and drought escape physiology

In herbaceous plants, the evolution of a drought escape strategy has been characterized as involving both life history and physiological components: early maturation, rapid completion of the life cycle, high photosynthetic and transpiration rates, and low WUE (Ludlow, 1989; Heschel and Rignios, 2005; Kooyers, 2015). The association between these components could be due to their joint, but independent, evolution or to the existence of genetic correlations that have constrained the available evolutionary trajectories. Several recent studies have provided support for the latter proposition (Geber and Dawson, 1990, 1997; Franks, 2011; Kimball *et al.*, 2013; Kenney *et al.*, 2014), and in *Arabidopsis thaliana* there is strong evidence that the associations between early flowering, high photosynthetic rate and low WUE are due to pleiotropy rather than linkage (McKay *et al.*, 2003, 2008; Lovell *et al.*, 2013).

In our study, selection for rapid maturation did not cause the correlated evolution of higher gas exchange rates or lower WUE. Although the maximum photosynthetic rate, A_{\max} and stomatal conductance g do appear to have some genetic basis in *C. unguiculata* (Jonas and Geber, 1999), gas exchange rates under natural conditions tend to be highly influenced by environmental factors and show low heritabilities (Geber and Griffen, 2003), potentially making differences in these variables between control and selected lines difficult to detect. This challenge should have been mitigated in our study by the common garden design, by statistical control of the effects of plot,

air temperature and measurement date, and by the use of family means as response variables. Nevertheless, variation among families was still high. This was true even for $\delta^{13}\text{C}$, which ought to be less affected than gas exchange rates or WUE by short-term environmental fluctuations. Differences between control and selected lines were not even consistent in direction across replicates for any of the four response variables, even though such consistency might have been expected if genetic correlations between maturation rate and gas exchange rates were present, but weak. Thus, although several loci are known to have pleiotropic effects on flowering time and WUE in *Arabidopsis* (McKay *et al.*, 2003, 2008; Kinoshita *et al.*, 2011; Lovell *et al.*, 2013), the observed response to selection for early flowering in *Clarkia* does not appear to have been mediated principally by selection on loci that also influence gas exchange rates. This is consistent with other evidence from *Arabidopsis* indicating the presence of multiple additional quantitative trait loci (QTLs) that independently affect flowering date or WUE (Juenger *et al.*, 2005; Lovell *et al.*, 2013). The main targets of selection in our experiment may well have been loci that mediate hormonal responses to photoperiod or the winter temperature regime, both of which have a major influence on flowering date (Van Dijk, 2009; Amasino, 2010).

Finally, our results suggest that the higher gas exchange rates observed in field populations of *C. exilis* compared with *C. unguiculata* (Mazer *et al.*, 2010; Dudley *et al.*, 2012) are not due to genetic differences arising from indirect selection on traits influencing gas exchange physiology when *C. exilis* was evolving a faster maturation rate and earlier flowering than its putative outcrossing common ancestor with *C. unguiculata*. Either the gas exchange rates evolved independently of life history traits in these lineages, or the observed phenotypic differences between the two taxa are due mainly to environmental causes. Evidence on the effects of seasonal drought on the evolution of drought escape physiology is currently mixed. Some studies have indicated that selection favours low WUE (*A/g*) only in moist environments (Sherrard and Maherali, 2006) or when drought conditions arrive relatively early in the life cycle (Heschel and Riginos, 2005). In contrast, Ivey and Carr (2012) found little evidence of selection on $\delta^{13}\text{C}$ under wet or dry conditions, whereas Kenney *et al.* (2014) found that selection for low WUE (measured by $\delta^{13}\text{C}$) was marginally stronger after imposition of a late season drought than under consistently well-watered conditions. Comparing such studies is difficult because terms such as ‘well-watered’ and ‘late season’ may mean different things for different plant species but, if the results of Heschel and Riginos (2005) or Sherrard and Maherali (2006) are also true of *Clarkia*, they may partly explain why early flowering, high gas exchange rates and low WUE in the largely selfing *C. exilis* are associated with its occupation of relatively cool, moist microenvironments (Vasek, 1958). Other selfing taxa derived from *C. unguiculata* (*C. tembloriensis* and ‘Caliente’ populations) occupy similar habitats (Vasek, 1968; Vasek and Sauer, 1971), although their gas exchange physiology has not yet been studied.

Conclusions

In *C. unguiculata*, both maturation rate (days to first flower) and anther–stigma distance responded significantly to artificial direct selection in the greenhouse after only 2–3 generations.

However, there was little evidence for the correlated evolution of maturation rate and floral traits associated with autogamous selfing (dichogamy and herkogamy), suggesting that the time limitation hypothesis, at least in its indirect selection scenario, does not apply to *Clarkia*. Selection for early flowering also did not cause the correlated evolution of the high gas exchange rates and low WUE associated with a drought escape physiology. Thus, the phenotypic associations between life history, mating system and gas exchange physiology traits seen across *Clarkia* taxa are most likely to have evolved through direct selection on each of these traits and their resulting joint, but independent evolution, rather than through indirect selection caused by genetic correlations between them.

SUPPLEMENTARY DATA

Supplementary data are available at <https://academic.oup.com/aob> and consist of the following. Table S1: summary statistics for each generation of selection. Table S2: raw data for floral traits in the field after three generations of selection for early flowering. Table S3: raw data for gas exchange traits in the field after three generations of selection for early flowering. Figure S1: raw data for days to first flower, node of first flower, anther–stigma distance and protandry of field-raised plants after three generations of selection for early flowering. Figure S2: raw data for gas exchange rates and water use efficiencies of field-raised plants after three generations of selection for early flowering.

ACKNOWLEDGEMENTS

We thank Dr Jennifer Cruise and undergraduates Kasey Diekmann, Rachael Eaton and Emily Novak for assistance with greenhouse work at UST, and undergraduates Bryce Rauterkus and Joel Kirskey for assistance with fieldwork in California. Numerous other undergraduates also helped with greenhouse work at both UST and UCSB. This work was supported by the National Science Foundation [OIS-0718253 to S.K.E. and A.S.V., and OIS-0718227 to S.J.M. and L.S.D.] and by the University of St. Thomas.

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