RESEARCH PAPER

Sex-specific phenotypic selection and geographic variation in gender divergence in a gynodioecious shrub

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ABSTRACT

In sexually polymorphic plant species the extent of gender divergence in floral morphology and phenology may be influenced by gender-specific selection patterns imposed by pollinators, which may change geographically. Distribution margins are areas where changes in the pollinator fauna, and thus variation in gender divergence of floral traits, are expected. We tested for pollination-driven geographic variation in the gender divergence in floral and phenological traits in the gynodioecious shrub *Daphne laureola*, in core and marginal areas differing in the identity of the main pollinator. Pollinators selected for longer corolla tubes in hermaphrodite individuals only in core populations, which in turn recorded higher fruit set. Consistent with these phenotypic selection patterns, gender divergence in flower corolla length was higher in core populations. Moreover, pollinators selected towards delayed flowering on hermaphrodite individuals only in marginal populations, where the two sexes differed more in flowering time. Our results support that a shift in main pollinators is able to contribute to geographic variation in the gender divergence of sexually polymorphic plant species.

INTRODUCTION

Improving our knowledge of the relative role of pollinators in floral evolution and plant diversification requires updating conventional phenotypic selection studies in the wild by focusing on populations with novel traits or in new environments where selective process should be particularly active (Harder & Johnson 2009). A rigorous demonstration of adaptive, pollinator-driven regional differentiation in floral traits of a plant species requires the verification of several components of selection, which are summarised in the following prediction: if variable pollinators are a major influence on floral diversification, then geographic variation of the pollinator fauna should impose geographic variation in selection on floral traits (Herrera *et al.* 2006). In this regard, ecological margins in plant distributions represent areas where populations are geographically more isolated and prone to changes in abundance and composition of pollinator faunas (Silva-Montellano & Eguiarte 2003; Moeller 2006; Stone & Jenkins 2008; Moeller *et al.* 2012).

The evolution of floral divergence in sexually polymorphic plant species includes the possibility of intralocus sexual conflict whenever the fitness optima of a certain floral trait differ among individuals of different sex (Chapman *et al.* 2003; van Doorn 2009; Cox & Calsbeek 2009). Furthermore, geographic variation in the magnitude of intralocus sexual conflict can be promoted by variable environmental conditions that redefine the adaptive landscape for one or both sexes (Delph & Herlihy 2011; Delph *et al.* 2011), and micro-evolutionary investigations

are needed on the extent of intraspecific variation in gender divergence of sexually polymorphic plants (Barrett & Hough 2013). Geographic variation in pollinator fauna composition can also lead to changes in gender-specific selection patterns imposed by pollinators on floral traits, resulting in geographic variation in the magnitude of intralocus sexual conflict and thus in the level of gender divergence of floral and/or phenological traits. Of particular interest in this context are gynodioecious plants where hermaphrodite and female individuals coexist within populations, which frequently exhibit pronounced gender divergence in floral traits, in striking contrast to modest divergence in vegetative traits (Ashman 2005). In gynodioecious species, flower size is almost universally larger in hermaphrodites than in females (Eckhart 1999; Shykoff *et al.* 2003). Gynodioecious plants usually present gender divergence in flowering phenology, where female plants flower earlier than hermaphrodites (Uno 1982; Ashman & Stanton 1991; Asikainen & Mutikainen 2005). Both flower size and flowering phenology may be largely influenced by the pollinator's selection patterns (Campbell 1989; Galen 1989; Johnston 1991; Conner *et al.* 1996). Therefore, we may expect that geographic variation in pollinator fauna can have a relevant role on the geographic variation of the gender divergence in floral and phenological traits of gynodioecious species, and thus on the intralocus sexual conflict in these phenotypic traits (Ashman 2006).

In this paper, we explored gender-related floral differentiation in core and marginal populations of the gynodioecious

shrub *Daphne laureola*, using an analysis of phenotypic selection by pollinators on floral traits following the protocol proposed in Herrera *et al.* (2006). *Daphne laureola* is an understorey shrub that presents gynodioecious populations with hermaphrodite and female individuals, and monomorphic populations made up exclusively of hermaphrodites (Alonso *et al.* 2007). We compare two disjunct groups of gynodioecious populations in the southern Iberian Peninsula that differ in the identity of the main pollinator (Alonso 2004; Castilla *et al.* 2011). Noteworthy, hermaphrodites of *D. laureola* present longer flowers and start their flowering earlier than females in continuous core populations (Alonso 2004, 2005), but the adaptive value and the magnitude of spatial variation of gender divergence have not been analysed in a broader geographic context. Specifically, we addressed the following questions: (i) are there contrasting selective regimes on individuals of different sex in continuous core and marginal disjunct populations; (ii) does the level of phenotypic gender divergence in floral and phenological traits differ between the two study regions; and (iii) is there differential phenotypic selection on floral and phenological traits matching the geographic variation in these traits?

MATERIAL AND METHODS

Study species and area

Daphne laureola L. (Thymelaeaceae) has a disjunct Palaearctic distribution associated with the main calcareous mountainous ranges of Europe and northern Africa, including the Baetic Ranges in southern Spain where this study was conducted (Castilla *et al.* 2012; Fig. S1). Within the Baetic Ranges, this species flowers from January to April, a period characterised by low temperatures, frequent rains and irregular snowfall. Each plant produces a large number of small, tubular, green-yellowish flowers aggregated into several compact inflorescences per stem. Plants of the two sexes are similar in size, flower and fruit production (Alonso & Herrera 2001; Castilla *et al.* 2011). Flowers present a tubular corolla with four lobes at the distal tip, they show reverse herkogamy (*i.e.* stigma below the anthers) that increases with the length of the corolla tube (cf. Medrano *et al.* 2005), and remain open continuously for approximately 1 month (Alonso 2004). The ovary has a single ovule, the style

is very short, the stigma is receptive even before the flower opens and remains receptive throughout the flowering period. Female flowers have vestigial stamens that do not produce pollen and have shorter corolla tubes. Hermaphrodite flowers bear eight stamens arranged in two whorls of four stamens each. Shortening exposure of *D. laureola* flowers to pollinators decreases fruit set in both female and hermaphrodite plants (Alonso 2004).

Across the Baetic Ranges, *D. laureola* presents a local continuous core region in the southeast, where populations are largely connected and the small pollen beetle *Meligethes elongatus* is the main pollinator (Alonso 2004; Castilla *et al.* 2012; Fig. S1). This pollen beetle forages actively for pollen and consequently prefers *D. laureola* hermaphrodite plants (Alonso 2004). The spatial isolation of populations and their genetic differentiation increases towards the western margin of the Baetic Ranges (Castilla *et al.* 2012; Fig. S1). Furthermore, the disjunct group of populations located on the western margin are characterised by a different pollinator fauna where the pollen beetle is absent and several bee and bumblebee species constitute the main floral visitors (Castilla *et al.* 2011). Therefore, in this study we considered the eastern populations of *D. laureola* as the local continuous core ('core region' hereafter) and populations at the western edge as a local disjunct margin ('marginal region' hereafter).

Geographic variation in phenotypic traits and phenotypic selection by pollinators

A subsample of six populations (three populations per region; Fig. S1, Table 1) was intensively studied to ascertain the possible role of pollinators as selective agents promoting geographic variation in gender divergence of floral and phenological traits. All the populations studied were gynodioecious. At the beginning of the study, we randomly marked 25 hermaphrodite and 15 female plants in each study population, to avoid potential artefacts due to uncontrolled variation on sample sex ratio. The proportion of female and hermaphrodite individuals was determined during the flowering period of 2007 by examining 100 randomly chosen individuals per population, and varied between 10% and 25% (Table 1). By the end of the flowering period, 12 flowers per marked plant were collected in each population (N = 2880 flowers). Flowers were preserved in FAA

Table 1. General features of the six *Daphne laureola* populations used in the analysis of selection on floral and phenological traits.

population	code	coordinates	region	altitude (m a.s.l.)	female frequency (%)
Valdecuevas	VCV	37°54′ N 2°52′ W	core	1380	25.5
Cañada del Espino	CDE	37°55′ N 2°53′ W	core	1575	20
Fuente Bermejo	FBJ	37°55′ N 2°50′ W	core	1513	21
Grazalema	GRZ	36°46′ N 5°25′ W	marginal	1229	10
Fuente Molina	FML	36°41′ N 5°1′ W	marginal	1380	20
Cañada de las Animas	CDA	36°42′ N 5°1′ W	marginal	1333	18.5

solution (2.5% formaldehyde, 2.5% acetic acid, 95% ethanol) until dissection and measurement. These flowers were used to characterise morphology (see below) and their styles stained to observe pollen tubes as the most proximate estimate of pre-zygotic pollination success (see Alonso 2005 for details). The proportion of fruits per flower was estimated using five inflorescences per marked plant ('fruit set' hereafter) and used as a proxy of maternal fitness.

Floral traits

The length and maximum width of the aperture of the flower tube, and the outer corolla diameter (estimated as mean length between the tips of two opposite lobes) of all collected flowers were measured under a dissecting microscope using an ocular micrometer (hereafter, respectively, named corolla length, aperture of tube and outer diameter).

Patterns in the phenological synchronisation of flowering can be described among- and within-individuals (Bolmgren 1998; Munguía-Rosas *et al.* 2011). As adaptive advantages of among-individual synchronisation could be confounded by within-individual patterns of synchronisation, we needed to separately investigate both levels of phenological synchronisation (Bolmgren 1998). We counted the number of open and closed flowers in five inflorescences per marked individual during peak flowering. We estimated flowering time and flowering asynchrony as two parameters related to among- and within-individual flowering synchronisation, respectively. Flowering time was estimated as the mean percentage of open flowers per inflorescence of each individual (*i.e.* larger values indicate earlier individuals). Flowering asynchrony was quantified through the coefficient of variation of the number of opened flowers among inflorescences within each marked individual.

All statistical analyses were performed using the SAS statistical package (SAS Institute, Cary, NC, USA). Differences in morphological and phenological traits between sexes and regions were analysed with generalised linear mixed models (Procedure GLIMMIX), with normal error distribution for morphology traits (corolla length, outer diameter and aperture of tube) and negative binomial error distribution for phenology traits (flowering time and flowering asynchrony). Sex and region effects were treated as fixed; population was treated as a random effect. When replicated at this level (*e.g.* morphological traits), individual plants within populations were treated as random effects.

Phenotypic selection on maternal fitness

The role of phenotypic selection by pollinators on the gender divergence should ideally be evaluated *via* female and male fitness. However, we focused on maternal fitness because in flowers with reverse herkogamy, such as *D. laureola*, a positive relationship between reception and export of pollen would be expected since no visitor will reach the stigma without touching the anthers. Further, there were some limitations to estimates of male fitness of hermaphrodites through either pollen export or genetic markers, because hermaphrodites of *D. laureola* present high selfing and inbreeding depression (Medrano *et al.* 2005). Thus pollen export alone does not accurately determine male fitness, whereas paternity analysis was not feasible because allozymes were not sufficiently variable

(cf. Medrano *et al.* 2005) and no highly variable co-dominant genetic markers are currently available for *D. laureola*.

Phenotypic selection on floral morphology and flowering phenology *via* its influence on pre-zygotic pollination success and maternal fitness were assessed using generalised linear models for each sex within region. We used the mean number of pollen tubes and mean fruit set (proportion of fruits per flower) as response variables related to pre-zygotic pollination success and individual maternal fitness, respectively. Pollen tubes represent the intermediate phase between pollen arrival and seed production that may better characterise the pollination phase without confounding the seed-filling stage in which pollination success interacts with resource availability (Alonso *et al.* 2012). In *D. laureola*, fruit set is positively correlated to seed production (maternal fitness) and constitutes a size-independent estimate of annual maternal fecundity (Castilla *et al.* 2011). We used relative values of both response variables, estimated by dividing the mean values per individual by the population means (Lande & Arnold 1983). All floral and phenological traits were standardised to mean 0 and variance 1, and were used as independent variables in models. Among-population variation in phenotypic selection on floral morphology and phenology was evaluated by testing the homogeneity of slopes of the relations of relative pollination success and maternal fitness to the measures of floral traits among populations, *i.e.* through analyses of population × corolla length, population × outer diameter, population × aperture of tube, population × flowering time and population × flowering asynchrony interactions (*e.g.* Strauss *et al.* 2005; Herrera *et al.* 2006). We first ran the full model with all of the interactions and then excluded the non-significant ones (Littell *et al.* 2006). For simplicity, we focused only on directional selection gradients and we did not assess quadratic terms in the phenotypic selection model, as this approach facilitates interpretation of population × traits interactions. We used partial residual plots to represent graphically the selection gradients per population and sex because they show the relationship between a given independent variable and the response variable, given that other independent variables are also in the model (Ryan 1997).

RESULTS

Intraspecific floral trait differentiation

Hermaphrodite flowers were longer and wider than female flowers. The magnitude of gender divergence in corolla length was lower in marginal populations ($F_{1,2392} = 30.63$, $P < 0.0001$; region × sex interaction; Fig. 1a). In particular, hermaphrodite flowers of marginal populations had shorter corollas than those of core populations (Fig. 1a), whereas female flowers were similar in both regions (Fig. 1a). Flowers of hermaphrodites had larger mean outer diameter than those of females ($F_{1,2321} = 174.08$, $P < 0.0001$; Fig. 1b). The magnitude of the difference between the two sexes was similar in core and marginal populations ($F_{1,2321} = 0.06$, $P = 0.8$; region × sex interaction; Fig. 1b). Flowers of hermaphrodites had larger mean aperture of tube than those of females in both core and marginal populations ($F_{1,2377} = 491.46$, $P < 0.0001$; Fig. 1c). The magnitude of the difference between the two sexes was similar between core and marginal populations ($F_{1,2377} = 2.22$, $P = 0.1$; region × sex interaction). In addition, flowers of the two sexes
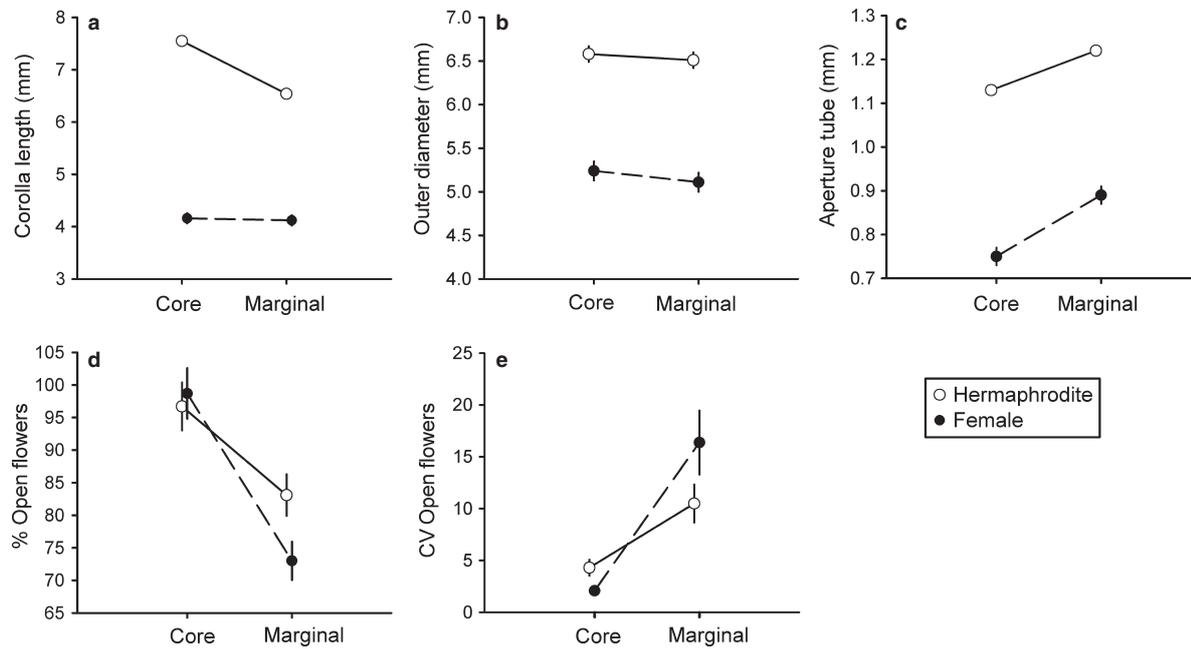
Fig. 1. Variation in average floral phenotypic traits between genders (hermaphrodites *versus* females) and regions (core *versus* marginal) of *Daphne laureola* in the southern Iberian Peninsula. Three morphological traits related to flower size were studied: (a) corolla length; (b) outer corolla diameter; and (c) aperture of tube. Additionally, two phenological traits were studied: percentage of open flowers during peak flowering, flowering time (d); and coefficient of variation of the number of open flowers during peak flowering, flowering asynchrony (e). Values plotted are the model-adjusted least-squares means ($\pm$SE).

had a larger mean aperture of the tube in marginal than in core populations ($F_{1,2377} = 52.95$, $P < 0.0001$; Fig. 1c).

Gender divergence in phenological traits differed in core and marginal populations of *D. laureola*. Sexes differed in the time of flowering in marginal populations, where females were delayed, but not in core populations ($F_{1,232} = 25.66$, $P < 0.0001$, interaction region $\times$ sex; Fig. 1d). Also, individuals in core populations had more open flowers per inflorescence at peak flowering than those of marginal populations. Within-plant asynchrony was higher in marginal populations, and the sign of gender divergence changed between regions ($F_{1,232} = 22.06$, $P < 0.0001$; interaction region $\times$ sex). Hermaphrodites presented more variation in the number of open flowers during peak flowering in core populations, in contrast to the marginal populations where females were more variable (Fig. 1e).

Phenotypic selection estimated from pollination success

Only some floral traits of hermaphrodite individuals were influential for the mean number of pollen tubes recorded per plant, our estimate of individual relative pollination success. In core populations, mean number of pollen tubes was significantly related to corolla length and aperture of tube in hermaphrodites, the level of statistic significance varying among populations (Table 2). In particular, the mean number of pollen tubes was positively related to corolla length in hermaphrodites of two core populations (CDE and VCV; Fig. 2 top panels). Furthermore, mean number of pollen tubes showed a marginally significant positive relationship with aperture of tube in hermaphrodites of VCV population (t = 1.85, $P = 0.07$; b = 0.14 $\pm$ 0.08). However, there was no significant relationship in the other two core populations ($P = 0.12$ and $P = 0.43$ for hermaphrodites of CDE and FBJ, respectively).

In addition, in marginal populations pollination success was not related to floral morphology but was larger in late-flowering hermaphrodite individuals (*i.e.* with later flowering time; Table 2). Neither floral morphological nor phenological traits were related to the mean number of pollen tubes in females of the two study regions (Table 2).

Phenotypic selection estimated from maternal fecundity

Again, phenotypic selection patterns were variable among sexes, populations and regions. In core populations, positive selection on variable corolla length among populations was detected in hermaphrodites (Table 3). As noted from pollination success (see previous section), hermaphrodites with higher fruit set also presented longer corollas in CDE and VCV populations, but not in FBJ (Fig. 2 lower panels). Females of all core populations exhibited positive selection on outer diameter (Table 3). Also in females, we detected selection on flowering asynchrony that varied among populations (Table 3). Higher fruit set was positively related to flowering asynchrony only in CDE (Fig. 3).

In marginal populations, we did not detect any phenotypic selection on traits of hermaphrodite plants (Table 3). However, flowering time and flowering asynchrony were under phenotypic selection in females of marginal populations, being the statistically significant variable among populations (Table 3). Fruit set was negatively related to flowering time and flowering asynchrony in females of GRZ population (Fig. 4). No phenotypic selection on flowering time was detected in CDA or in FML (t = 0.19, $P = 0.85$ and t = −0.71, $P = 0.48$). Similarly, flowering asynchrony was not related to fruit set in CDA and FML (t = −0.23, $P = 0.82$ and t = −0.63, $P = 0.53$).

Table 2. ANCOVA analyses comparing patterns of selection by pollinators on floral traits in females and hermaphrodites of core and marginal populations of *Daphne laureola*.

effect	df	core hermaphrodites			marginal females			marginal females			marginal females		
		$F_{(62)}$	P	b (SE)	$F_{(33)}$	P	b (SE)	$F_{(62)}$	P	b (SE)	$F_{(36)}$	P	b (SE)
CL	1	**6.16**	**<0.05**	**0.20 (0.08)**	0.41	0.52	0.09 (0.14)	0.00	0.96	0.01 (0.09)	0.12	0.74	−0.04 (0.11)
OD	1	0.98	0.33	0.05 (0.05)	0.07	0.79	−0.04 (0.14)	0.28	0.60	0.05 (0.10)	0.21	0.65	0.05 (0.12)
AT	1	0.50	0.48	0.14 (0.08)	0.03	0.87	0.03 (0.15)	0.44	0.51	0.07 (0.10)	0.47	0.50	0.08 (0.11)
FT	1	0.48	0.49	0.07 (0.11)	0.93	0.34	−0.25 (0.26)	**5.30**	**<0.05**	**−0.25 (0.11)**	1.30	0.26	−0.14 (0.12)
FA	1	0.02	0.90	0.01 (0.10)	1.12	0.30	−0.22 (0.21)	0.05	0.82	−0.03 (0.11)	2.16	0.15	0.16 (0.11)
Pop	2	0.79	0.46		0.41	0.66		0.59	0.56		0.05	0.95	
CL × Pop	2	**3.31**	**<0.05**										
AT × Pop	2	**3.33**	**<0.05**										

Dependent variable was the relative mean number of pollen tubes; independent variables were standardised to mean 0 and variance 1. Significant relationships ($P \leq 0.05$) are in bold. Marginally significant relationships ($0.05 < P < 0.1$) are in italics. CL, OD, AT, FT and FA refer to corolla length, outer diameter, aperture of corolla tube, flowering time and flowering asynchrony, respectively. We first ran the model with all of the interactions and then excluded the non-significant ones. Only linear selection gradients were considered (see Material and Methods). Results show the standardised selection coefficient (b) and its standard error mean (SE).
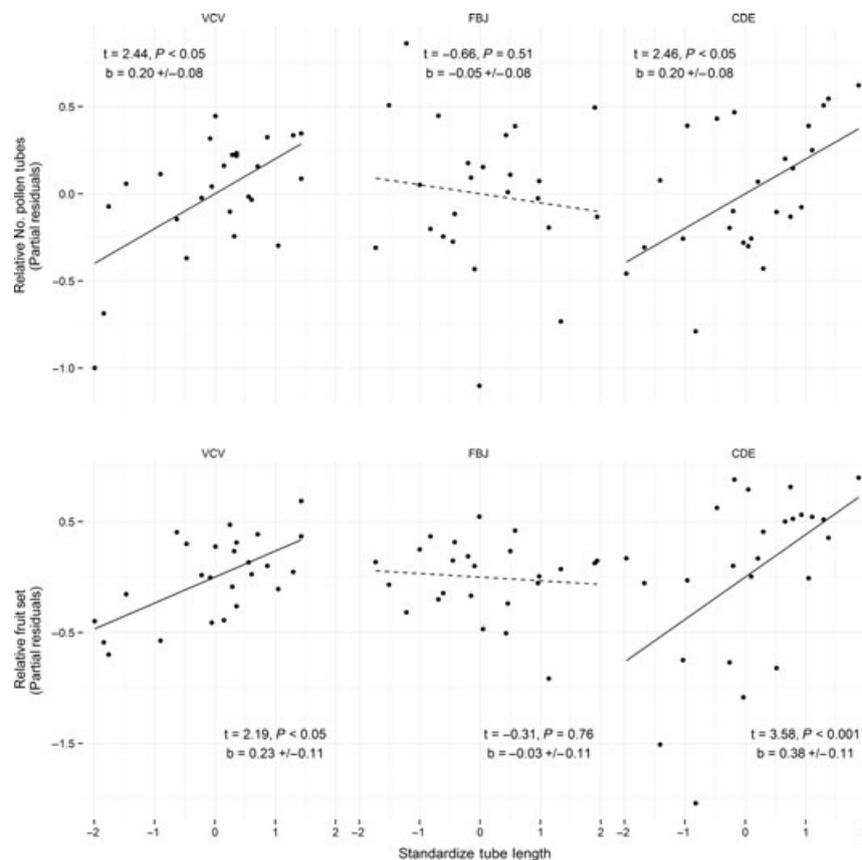

Fig. 2. Standardised linear phenotypic selection gradients for length of corolla, based on number of pollen tubes per style, as an estimate of pollination success (upper panels), and fruit set, as an estimate of maternal fecundity (bottom panels), in hermaphrodite individuals of core populations of *Daphne laureola*. We represent graphically the partial residuals of the dependent variable; independent variable was standardised to mean 1 and variance 0. Solid and dashed lines indicate significant and non-significant relationships, respectively. Note that scale in the vertical axis is different in upper and bottom panels.

DISCUSSION

Most micro-evolutionary investigations on the extent of intra-specific variation in gender divergence in sexually polymorphic plants have involved a limited sample of populations, and thus currently little is known about the patterns of geographic variation in the magnitude of the divergence (Barrett & Hough 2013). Our study attempts to reduce this gap, but notwithstanding, our results are also congruent with a recent review concluding that phenotypic selection on floral and plant traits

Table 3. ANCOVA analyses comparing patterns of selection on floral traits in females and hermaphrodites of core and marginal populations of *Daphne laureola*.

effect	df	core hermaphrodites			core females			marginal hermaphrodites			marginal females		
		$F_{(64)}$	P	b (SE)	$F_{(34)}$	P	b (SE)	$F_{(62)}$	P	b (SE)	$F_{(32)}$	P	b (SE)
CL	1	**9.59**	**<0.01**	**0.23 (0.11)**	0.34	0.56	0.04 (0.08)	0.00	0.96	0.002 (0.05)	*3.50*	*0.07*	*0.15 (0.08)*
OD	1	0.13	0.72	−0.02 (0.06)	**8.49**	**<0.01**	**0.24 (0.08)**	0.11	0.74	−0.02 (0.05)	0.22	0.64	0.04 (0.09)
AT	1	0.05	0.83	−0.01 (0.06)	2.58	0.12	0.17 (0.10)	0.32	0.57	0.03 (0.05)	0.65	0.42	−0.06 (0.08)
FT	1	0.19	0.66	−0.06 (0.14)	*3.03*	*0.09*	*0.41 (0.24)*	0.39	0.53	0.04 (0.06)	**5.13**	**<0.05**	**−0.56 (0.18)**
FA	1	0.03	0.87	−0.02 (0.14)	1.72	0.20	0.09 (0.21)	0.03	0.86	−0.01 (0.06)	**12.84**	**<0.01**	**−0.91 (0.20)**
Pop	2	1.39	0.26		0.25	0.78		0.34	0.71		*2.59*	*0.09*	
CL × Pop	2	**4.02**	**<0.05**										
FT × Pop	2										**3.34**	**<0.05**	
FA × Pop	2				**5.15**	**<0.05**					**7.13**	**<0.01**	

Dependent variable was relative fruit set, as an estimate of maternal fecundity; independent variables were standardised to mean 0 and variance 1. Significant relationships ($P \leq 0.05$ or better) are in bold. Marginary significant relationships ($0.05 < P < 0.01$) are CL, OD, AT, FT and FA refer to corolla length, outer diameter, aperture of corolla tube, flowering time and flowering asynchrony, respectively. We first ran the model with all of the interactions and then excluded the non-significant ones. Only linear selection gradients were considered (see Material and Methods). Results show the standardised selection coefficient (b) and its standard error mean (SE).
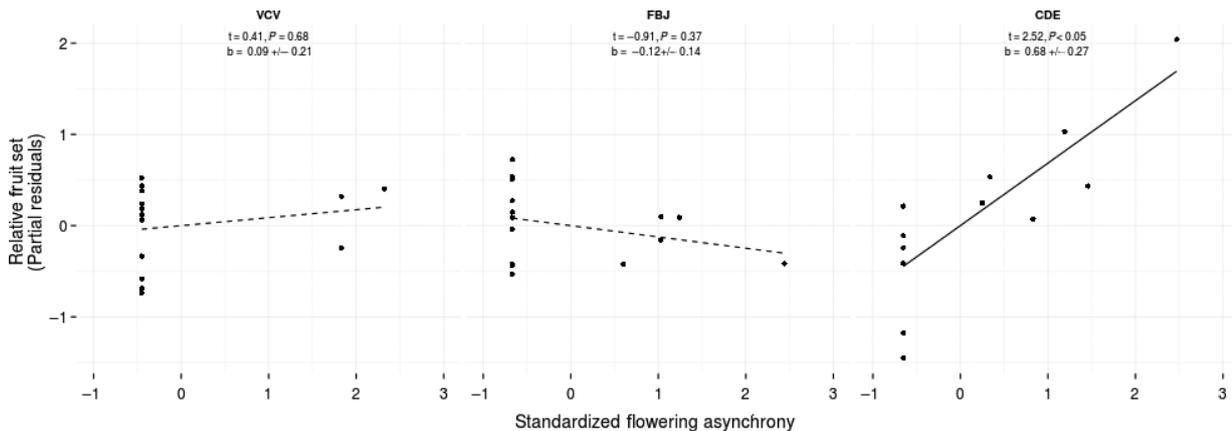

Fig. 3. Standardised linear phenotypic selection gradients for flowering asynchrony (FA) in female individuals of core populations of *Daphne laureola*. Flowering asynchrony between inflorescences of the same individual was estimated through the coefficient of variation of the number of open flowers per inflorescence (N = 5 per plant) during the population flowering peak. Dependent variable was the relative fruit set, estimated by dividing mean values per individual by population means. We represent graphically the partial residuals of the dependent variable.

related to pollination acts sporadically within populations and inconsistently among populations (Harder & Johnson 2009). Indeed, corolla length was the only trait among all studied floral traits that resulted in congruent phenotypic selection patterns based on both pre-zygotic pollination success and post-pollination maternal fitness of individuals, indicating positive selection on *D. laureola* hermaphrodites at the core region. Thus, we focus our discussion on this morphological trait, frequently associated with pollinator attraction and dimorphic in many gynodioecious species (Delph *et al.* 1996; Shykoff *et al.* 2003).

Available data support geographic variation in the extent of gender divergence in floral size and morphology in some species (Kohorn 1995; Delph *et al.* 2002; Delph & Bell 2008). In *D. laureola*, corolla length of hermaphrodite flowers varied between study regions, suggesting that its size was at least partly determined by its attraction function and not only by developmental constraints (Delph *et al.* 1996). Changes in

composition of the pollinator community can lead to different patterns of phenotypic selection on floral traits (Herrera *et al.* 2006; Moeller 2006), however few studies have investigated the role of pollinators as causal agents of gender divergence in floral traits of gynodioecious species (*e.g.* Ashman & Diefenderfer 2001; Case & Ashman 2009). We reported a shift from a pollinator fauna made up almost exclusively of a pollen beetle species in core populations of *D. laureola* to another constituted by several bumblebee and bee species in marginal populations where the fruit set was higher (Castilla *et al.* 2011). Now, our phenotypic selection analyses based on pre-zygotic pollination success support different phenotypic selection gradients *via* pollination in core and marginal populations of *D. laureola*. Corolla length was positively and significantly selected *via* pollination in hermaphrodites of two core populations, but in none of the marginal populations, nor in females. Furthermore, these phenotypic selection patterns had consequences for the maternal fitness of the hermaphrodites, with individuals
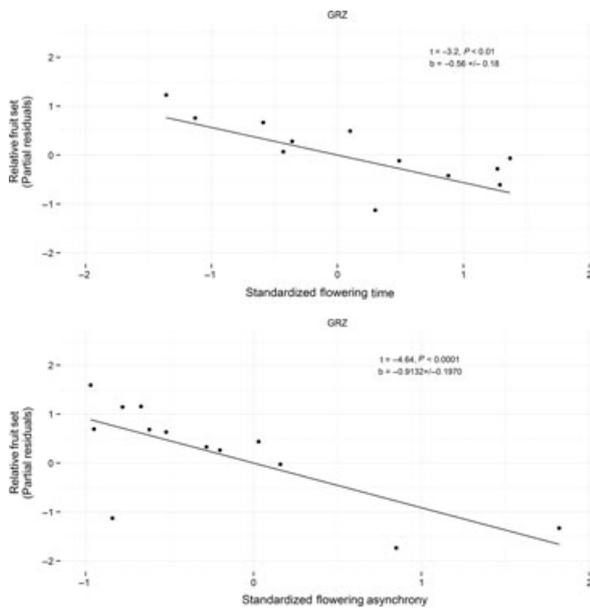
Fig. 4. Standardised linear phenotypic selection gradients for flowering time and flowering asynchrony (FT and FA, respectively) in female individuals of the marginal population GRZ. Significant relationships between these phenological variables and relative fruit set were only found in females of this marginal population (see Results). Flowering time was estimated as the mean percentage of open flowers per inflorescence in each individual during the population peak flowering. Flowering asynchrony was estimated through the variation coefficient of the number of open flowers per inflorescence in each individual during the population flowering peak. Dependent variable was relative fruit set, estimated by dividing mean values per individual by population mean. We represent graphically the partial residuals of the dependent variable.

having longer tubes having higher fruit set only in these two populations. These patterns were consistent with the geographic variation observed for this floral trait. Divergence in corolla length was higher in core populations, where pollinators exerted positive phenotypic selection on this trait in hermaphrodites, but not in female plants. Selection could thus be interpreted as a process contributing to increased divergence at locations where corolla length and its variation *vis-à-vis* gender were already higher. Reduced gender divergence in the absence of phenotypic selection suggests that intralocus sexual conflict does not largely constraint floral evolution for this particular trait and species (Delph & Herlihy 2011; Delph *et al.* 2011; Barrett & Hough 2013).

Phenotypic selection towards a larger corolla in hermaphrodites of core populations could also be related to the mating system. In core populations, hermaphrodites receive much larger stigmatic pollen loads than females (Alonso *et al.* 2012), and adults of the pollen beetle *M. elongatus* promote extensive selfing in hermaphrodite offspring (Medrano *et al.* 2005). A longer corolla in hermaphrodites of core populations leads to increased herkogamy in their flowers (cf. Medrano *et al.* 2005), and could eventually alleviate selfing of the offspring and/or increase the diversity of pollen donors siring seeds, as shown for other self-compatible plants with longer styles (Karron *et al.* 1997; Medrano *et al.* 2012). The lower frequency of geitonogamous pollination by Hymenopteran poll-

inators at marginal populations could explain the absence of positive selection on this trait. Further studies analysing the differences in outcrossing rates in core and marginal populations of *D. laureola* and its relationship to corolla length will allow testing of this prediction. Alternatively, reduced ability of Hymenopteran pollinators to discriminate plants of different sexes could explain the absence of differences in the phenotypic selection patterns in females and hermaphrodites of marginal populations.

Less attention has been paid to gender divergence in phenological aspects (Bolmgren 1998; Munguía-Rosas *et al.* 2011). We might predict that the mating system of hermaphrodites could vary in relation to within- and among-plant synchronisation in flowering. We found consistent across-population phenotypic selection at the level of pollen tubes only in hermaphrodite individuals of marginal populations, where delayed flowering hermaphrodites had more pollen tubes. In marginal populations, we found larger gender divergence in flowering time than in core populations, where hermaphrodites started flowering earlier than females. Thus, selection by pollinators could be reducing a maladaptive gender divergence in flowering phenology in marginal populations of *D. laureola*.

CONCLUDING REMARKS

Marginal regions in plant distributions often present large fragmentation and thus constitute natural areas where shifts in pollinator fauna are feasible. These shifts in the composition of pollinator fauna may have important consequences for selection patterns by pollinators in plant populations, particularly in sexually polymorphic plant species. Results of the present study support this prediction for *D. laureola* populations in the southern Iberian Peninsula. We found that gender-specific selection patterns by pollinators on flower length and flowering time were different in core and marginal populations of *D. laureola*, congruent with a geographic variation in the extent of gender divergence in such traits. The results of the present study further support interest in marginal populations as natural areas where changes in the evolutionary dynamics of plant–pollinator interactions can be expected.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Distribution of *Daphne laureola* populations in the Baetic Ranges (southern Spain), and location of the core and marginal populations defined in this study, here denoted by filled circles and black triangles, respectively. The dashed boxes indicate the approximate locations of core and marginal

populations used in the analysis of selection on floral and phenological traits. Intervals of altitude (m a.s.l.) in the study area are represented with grey scale (see legend).

Table S1. Relationship between fruit set and fruit production per plant in each sex within each study population.

REFERENCES

Alonso C. (2004) Early blooming's challenges: extended flowering season, diverse pollinator assemblage and the reproductive success of gynodioecious *Daphne laureola*. *Annals of Botany*, **93**, 61–66.

Alonso C. (2005) Pollination success across an elevation and sex ratio gradient in gynodioecious *Daphne laureola*. *American Journal of Botany*, **92**, 1264–1269.

Alonso C., Herrera C.M. (2001) Neither vegetative nor reproductive advantages account for high frequency of male-steriles in southern Spanish gynodioecious *Daphne laureola* (Thymelaeaceae). *American Journal of Botany*, **88**, 1016–1024.

Alonso C., Mutikainen P., Herrera C.M. (2007) Ecological context of breeding system variation: sex, size and pollination in a (predominantly) gynodioecious shrub. *Annals of Botany*, **100**, 1547–1556.

Alonso C., Herrera C.M., Ashman T.-L. (2012) A piece of the puzzle: a method for comparing pollination quality and quantity across multiple species and reproductive events. *New Phytologist*, **193**, 532–542.

Ashman T.-L. (2005) The limits on sexual dimorphism in vegetative traits in a gynodioecious plant. *American Naturalist*, **166**, S5–S16.

Ashman T.-L. (2006) The evolution of the separate sexes: a focus on the ecological context. In: Barrett S.C.H., Harder L.D. (Eds), *Ecology and evolution of flowers*. Oxford University Press, Oxford, UK, pp 99–122.

Ashman T.-L., Diefenderfer C. (2001) Sex ratio represents a unique context for selection on attractive traits: consequences for the evolution of sexual dimorphism. *American Naturalist*, **157**, 334–347.

Ashman T.-L., Stanton M.L. (1991) Seasonal variation in pollination dynamics of sexually dimorphic *Sidalcea oregano* ssp. *spicata* (Malvaceae). *Ecology*, **72**, 993–1003.

Asikainen E., Mutikainen P. (2005) Preferences of pollinators and herbivores in gynodioecious *Geranium sylvaticum*. *Annals of Botany*, **95**, 879–886.

Barrett S.C.H., Hough J. (2013) Sexual dimorphism in flowering plants. *Journal of Experimental Botany*, **64**, 67–82.

Bolmgren K. (1998) The use of synchronization measures in studies of plant reproductive phenology. *Oikos*, **82**, 411–415.

Campbell D.R. (1989) Measurements of selection in a hermaphrodite plant: variation in male and female pollination success. *Evolution*, **43**, 318–334.

Case A.L., Ashman T.-L. (2009) Resources and pollinators contribute to population sex-ratio bias and pollen limitation in *Fragaria virginiana* (Rosaceae). *Oikos*, **118**, 1250–1260.

Castilla A.R., Alonso C., Herrera C.M. (2011) Exploring local borders of distribution in the shrub *Daphne laureola*: individual and population traits. *Acta Oecologica*, **37**, 269–276.

Castilla A.R., Alonso C., Herrera C.M. (2012) Genetic structure of the shrub *Daphne laureola* across the Baetic Ranges, a Mediterranean glacial refugium and biodiversity hotspot. *Plant Biology*, **14**, 515–524.

Chapman T., Arnqvist G., Bangham J., Rowe L. (2003) Sexual conflict. *Trends in Ecology & Evolution*, **18**, 41–47.

Conner J.K., Rush S., Jennetten P. (1996) Measurements of natural selection on floral traits in wild radish (*Raphanus raphanistrum*). I. Selection through lifetime female fitness. *Evolution*, **50**, 1127–1136.

Cox R.M., Calsbeek R. (2009) Sexually antagonistic selection, sexual dimorphism, and the resolution of intralocus sexual conflict. *American Naturalist*, **173**, 176–187.

Delph L.F., Bell D. (2008) A test of the differential-plasticity hypothesis for variation in the degree of sexually dimorphism in *Silene latifolia*. *Evolutionary Ecology Research*, **10**, 61–75.

Delph L.F., Herlihy C.R. (2011) Sexual fecundity and viability selection on flower size and number in a sexually dimorphic plant. *Evolution*, **66**, 1154–1166.

Delph L.F., Galloway L.F., Stanton M.L. (1996) Sexual dimorphism in flower size. *American Naturalist*, **148**, 299–320.

Delph L.F., Knapczyk F., Taylor D.R. (2002) Among-population variation and correlations in sexually dimorphic traits of *Silene latifolia*. *Journal of Evolutionary Biology*, **15**, 1011–1020.

Delph L.F., Andicoechea J., Steven J.C., Herlihy C.R., Scarpino S.V., Bell D.L. (2011) Environment-dependent intralocus sexual conflict in a dioecious species. *New Phytologist*, **192**, 542–552.

van Doorn G.S. (2009) Intralocus sexual conflict. *Annals of the New York Academy of Sciences*, **1168**, 52–71.

Eckhart V.M. (1999) Sexual dimorphism in flowers and inflorescences. In: Geber M.A., Dawson T.E., Delph L.F. (Eds), *Gender and sexual dimorphism in flowering plants*. Springer, Berlin, Germany, pp 123–148.

Galen C. (1989) Measuring pollinator-mediated selection on morphometric floral traits: bumblebees and the alpine sky pilot, *Polemonium viscosum*. *Evolution*, **43**, 882–890.

Harder L.D., Johnson S.D. (2009) Darwin's beautiful contrivances: evolutionary and functional evidence for floral adaptation. *New Phytologist*, **183**, 530–545.

Herrera C.M., Castellanos M.C., Medrano M. (2006) Geographical context of floral evolution: towards an improved research programme in floral diversification. In: Barrett S.C.H., Harder L.D. (Eds), *Ecology and evolution of flowers*. Oxford University Press, Oxford, UK, pp 278–294.

Johnston M.O. (1991) Natural selection on floral traits in two species of *Lobelia* with different pollinators. *Evolution*, **45**, 1468–1479.

Karron J.D., Jackson R.T., Thumser N.N., Schlicht S.L. (1997) Outcrossing rates of individual *Mimulus ringens* genets are correlated with anther–stigma separation. *Heredity*, **79**, 365–370.

Kohorn L.U. (1995) Geographic variation in the occurrence and extent of sexual dimorphism in a dioecious shrub, *Simmondsia chinensis*. *Oikos*, **74**, 137–145.

Lande R., Arnold S.J. (1983) The measurement of selection on correlated characters. *Evolution*, **37**, 1210–1226.

Littell R.C., Milliken G.A., Stroup W.W., Wolfinger R.D., Schabenberger O. (2006) *SAS for mixed models*, 2nd edition. SAS Institute, Cary, NC, USA.

Medrano M., Alonso C., Herrera C.M. (2005) Mating system, sex ratio, and persistence of females in the gynodioecious shrub *Daphne laureola* L. (Thymelaeaceae). *Heredity*, **94**, 37–43.

Medrano M., Requerey R., Karron J.D., Herrera C.M. (2012) Herkogamy and mate diversity in the wild daffodil *Narcissus longispathus*: beyond the selfing–outcrossing paradigm in the evolution of mixed mating. *Plant Biology*, **14**, 801–810.

Moeller D.A. (2006) Geographic structure of pollinator communities, reproductive assurance, and the evolution of self-pollination. *Ecology*, **87**, 1510–1522.

Moeller D.A., Geber M.A., Eckhart V.M., Tiffin P. (2012) Reduced pollinator service and elevated pollen limitation at the geographic range limit of an annual plant. *Ecology*, **93**, 1036–1048.

Munguía-Rosas M.A., Ollerton J., Parra-Tabla V., De-Nova J.A. (2011) Meta-analysis of phenotypic selection on flowering phenology suggests that early flowering plants are favoured. *Ecology Letters*, **14**, 511–521.

Ryan T. (1997) *Modern regression methods*. John Wiley & Sons, New York, NY, USA.

Shykoff J.A., Kolokotronis S., Collin C.L., López-Villavicencio M. (2003) Effects of male sterility on reproductive traits in gynodioecious plants: a meta-analysis. *Oecologia*, **135**, 1–9.

Silva-Montellano A., Eguiarte L.E. (2003) Geographic patterns in the reproductive ecology of *Agave lechuguilla* (Agavaceae) in the Chihuahuan desert. I. Floral characteristics, visitors and fecundity. *American Journal of Botany*, **90**, 377–387.

Stone J.L., Jenkins E.G. (2008) Pollinator abundance and pollen limitation of a solanaceous shrub at premontane and lower montane sites. *Biotropica*, **40**, 55–61.

Strauss S.Y., Sahli H., Conner J.K. (2005) Toward a more trait-centered approach to diffuse (co)evolution. *New Phytologist*, **165**, 81–89.

Uno G.E. (1982) Comparative reproductive biology of hermaphroditic and male-sterile *Iris douglasiana* Herb. (Iridaceae). *American Journal of Botany*, **69**, 818–823.