Research Article

Inflorescence characteristics as function-valued traits: Analysis of heritability and selection on architectural effects

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Abstract Production of multiple flowers in inflorescences allows the reproductive phenotypes of individual plants to include systematic among-flower variation, which could be adaptive. Systematic trait variation within inflorescences could arise from resource competition among flowers, or be a developmentally determined feature of flower position, regardless of resource dynamics. The latter, architectural effect typically manifests as continuous floral variation within inflorescences. For architectural effects to be adaptive, floral trait variation among individuals must covary with reproductive performance and be heritable. However, heritability and phenotypic selection on gradients of variation cannot be estimated readily with traditional statistical approaches. Instead, we advocate and illustrate the application of two functional data analysis techniques with observations of Delphinium glaucum (Ranunculaceae). To demonstrate the parameters-as-data approach we quantify heritability of variation in anthesis rate, as represented by the regression coefficient relating daily anthesis rate to inflorescence age. SNP-based estimates detected significant heritability ($h^2 = 0.245$) for declining anthesis rate within inflorescences. Functional regression was used to assess phenotypic selection on anthesis rate and a floral trait (lower sepal length). The approach used spline curves that characterize within-inflorescence variation as functional predictors of a plant's fruit set. Selection on anthesis rate varied with inflorescence age and the duration of an individual's anthesis period. Lower sepal length experienced positive selection for basal and distal flowers, but negative selection for central flowers. These results illustrate the utility and power of functional-data analyses for studying architectural effects and specifically demonstrate that these effects are subject to natural selection and hence adaptive.

Key words: architectural effects, functional data analysis, heritability, inflorescence display, selection.

1 Introduction

Reproduction by individual angiosperms is typically diversified among multiple flowers, which are usually aggregated in inflorescences (Harder & Prusinkiewicz, 2013). Production of multiple flowers allows for trait variation among flowers, in which case a plant's phenotype for a given floral trait includes its mean, variation and covariation within inflorescences (Diggle, 2003; Herrera, 2009). As development of all flowers within an inflorescence is controlled by the same genome, floral variation could simply represent phenotypic plasticity, and so be determined by ecological circumstance, such as resource competition among early- and late-pollinated flowers (reviewed by Diggle, 2003). However, a systematic gradient in floral traits within an inflorescence could also be subject to controlled variation in gene expression and floral development, rendering it an intrinsic feature of flower position (Diggle, 2003, 2014). To the extent that architectural effects confer functional benefits (e.g., Brunet & Charlesworth, 1995; Kudo & Kasagi, 2004; Cameron-Inglis, 2016) and differ genetically among individuals they could be subject to natural selection.

Statistical methods typically applied in analyses of natural selection either cannot be used straightforwardly, or are inappropriate for architectural effects. The heritable basis for a gradient of within-individual variation cannot be addressed using traditional quantitative genetics, as trait means do not capture patterns of systematic variation (Kingsolver et al., 2001; Stinchcombe et al., 2012). Similarly, standard regression methods for quantifying phenotypic selection (e.g., Lande & Arnold, 1983) are not applicable if the trait of interest is a gradient of within-individual variation. In particular, fitness cannot be assessed for individual flower positions, as fitness is a plant-level trait and, again, a mean cannot represent withinindividual variation and co-variation. To deal with overall trait variation in phenotypic selection studies, Herrera (2009) proposed including an individual's mean and variance as independent variables in regression analyses and demonstrated several cases of phenotypic selection on variance, including some for which selection on the mean was not evident (also see Arceo-Gómez et al., 2017). However, this approach does not account for the systematic within-individual variation that characterizes architectural effects.

A relatively new body of statistical methods, known as functional data analysis (Ramsay & Silverman, 1997, 2005), provides techniques for addressing these problems. These methods treat patterns of variation as functions, which are then represented in statistical models as equivalent to dependent and/or independent variables. In the context of within-individual variation, such patterns are known as function-valued traits (Kingsolver et al., 2001). Functional data analysis can be applied directly in the analysis of many aspects of organismal biology, including growth (Kingsolver et al., 2001) and continuous phenotypic responses to environmental change (Stinchcombe et al., 2012).

In this manuscript, we illustrate the utility of these techniques for analyzing heritability of, and phenotypic selection on, systematic gradients of floral variation within inflorescences. We begin with a brief overview of relevant approaches for assessing both heritability and the relation of a single fitness measure to function-valued traits. We then illustrate these methods with examples drawn from our studies of *Delphinium glaucum* S. Watson (Ranunculaceae), which exhibits gradients in various floral traits within its vertical racemes (Gallwey, 2011; Ishii & Harder, 2012). Our intention here is to introduce the general application of methods to the study of inflorescences, rather to provide a detailed interpretation of the nature of selection on *D. glaucum* inflorescences.

2 Brief Overview of the Analysis of Function-Valued Traits

We illustrate two approaches to the analysis of functionvalued traits: parameters as data, and functional regression. As the name suggests, the parameters-as-data approach uses estimates of a parametric model that characterize an individual's variation in a trait of interest in standard analyses of heritability (or evolvability) or phenotypic selection (Hernandez, 2015). As an example, Herrera (2009) used the variance of within-plant variation in floral traits as an independent variable in regressions assessing sources of among-plant variation in fruit number. Analysis of architectural effects will be more complex, because within-individual variation must be summarized with estimates of parameters that characterize the relation of floral-trait values to flower position. In the simplest case, this relation could be represented by the slope of the linear regression (perhaps in link space for a generalized linear model) for each individual. More generally, parameter estimates for nonlinear models may need to be estimated. The suitability of this approach will depend on identification of an appropriate parametric model to represent within-inflorescence variation. Ideally, the use of parameter estimates in subsequent analyses would account for the statistical uncertainty associated with these estimates. For example, regression analysis to assess phenotypic selection could be weighted by $1/s^2$, where $s^2 = (n \cdot SE)^2$ is the sampling variance of a parameter estimate, which can be calculated from the sample size (n) and standard error associated with the estimate (SE). Below, we use this approach to estimate heritability in changes in the number

of new flowers opened per day (anthesis rate) as flowering progresses within inflorescences.

Functional regression is specifically relevant to the analysis of phenotypic selection. As a method within the larger body of techniques for functional data analysis it uses non-parametric curve-fitting methods to characterize variation in a functionvalued trait (Ramsay & Silverman, 2005; Morris, 2015). These methods typically involve basis-function expansions using splines, Fourier series, or Legendre polynomials. Below, we use penalized splines (p-splines: Goldsmith et al., 2011) to depict variation in lower sepal length among flower positions within D. glaucum inflorescences. Analysis of phenotypic selection involves regressing single "fitness" measurements for individual plants (e.g., total fruit or seed number) against the fitted function (e.g., spline) for within-plant variation and perhaps additional plant-level covariates (scalar on function regression: Ramsay & Silverman, 2005). Rather than estimating a single (partial) regression coefficient for function-valued traits, functional regression estimates the relation of the regression coefficient for the effect of the function-valued trait (e.g., lower sepal length) on fitness (e.g., fruit number) to the underlying position on the trait gradient (e.g., flower position). This approach accounts for within-individual variation and covariation, including the associated measurement uncertainty.

3 Material and Methods

Delphinium glaucum is a hermaphroditic perennial that produces 5–75 purple, zygomorphic flowers along racemose inflorescences. As for many other bumble-bee pollinated angiosperms, *D. glaucum* flowers open sequentially from bottom to top (acropetally). After flower opening, anthers dehisce and present pollen during a 2–4 day male phase, followed by the onset of stigma receptivity during a 2–3 day female phase (Ishii & Harder, 2012). We studied a population of approximately 100 *D. glaucum* plants on Moose Mountain (50°54′00.5″N, 114°45′17.1″W), Alberta, Canada.

3.1 Data collection

Here, we report results for two function-valued traits, one phenological and one morphological, measured separately during 2014 and 2015. During 2014, we recorded the number of new flowers that opened daily on each plant during the entire flowering season (middle July to middle August) for 110 plants. In this case, anthesis rate was the trait of interest, which could vary with the number of days elapsed since the first flower opened on an inflorescence (inflorescence age). For *D. glaucum*, anthesis rate generally declines with inflorescence age (Gallwey, 2011). Total seed number was used as the measure of female fitness.

During 2015, we studied 64 individuals, measuring linear floral traits for seven evenly-spaced flowers along each inflorescence. Here, we consider variation in the length of a lower sepal, measured with digital calipers, which decreases from basal to distal flowers (Ishii & Harder, 2012). We recorded the position of each sampled flower from the bottom of the inflorescence as relative flower position (1–7), which identified which seventh of the inflorescence a flower represented. Fruit set was recorded for all flower positions and total fruit production was used as a measure of a plant's female success.

For plants sampled during 2014, we performed genotypingby-sequencing to acquire sufficient genetic markers for accurate measurement of genetic relatedness and heritability. DNA was extracted from dried leaf tissue using a modified cetyltrimethylammonium bromide (CTAB) technique (Doyle & Doyle, 1987) with RNAse A (ThemoFischer Scientific). Genomic library preparation was performed at the Institut de Biologie Intégrative et des Systèmes (IBIS, University of Laval, Laval, Quebec) according to the protocol of Poland et al. (2012). Libraries were then sequenced on an Illumina HiSeq2000 at the Genome Quebec Innovation Centre (McGill University, Quebec). From the resulting 187 million sequence reads, we identified 10458 variable SNP loci using the STACKS v 9.2 pipeline (Catchen et al., 2011, 2013).

3.2 Data analysis

To illustrate the overall relation of lower sepal length to relative flower position and of anthesis rate to inflorescence age, we plotted variation in the relevant least-squares means (\pm SE). These means were estimated based on generalized linear mixed models, as implemented in the glimmix procedure of SAS/STAT 14.1 (SAS Institute Inc., 2015). Lower sepal length was modeled with a normal distribution and identity link function, whereas anthesis rate was modeled with a negative binomial distribution and In link function. Both analyses accounted for random variation among plants and correlated responses within plants.

As an example of the parameters-as-data approach, we assessed the heritability of the regression coefficient (*c*) from plant-specific generalized linear models relating anthesis rate to ln(inflorescence age) for the 110 plants sampled during 2014. The generalized linear models were fit using the glimmix procedure of SAS/STAT 14.1 (SAS Institute Inc., 2015), with a negative binomial distribution and ln link function. We determined genetic relatedness among the sampled plants with the program GCTA v 1.26 (Yang et al., 2011). This program used restricted maximum likelihood to determine the amount of among-plant variation in *c* that was associated with genotypic variation in the 10458 SNPs. To implement this analysis, we first constructed a binary PED file with the PLINK whole genome tool (Purcell et al., 2007).

We assessed phenotypic selection on gradients of variation in lower sepal length and anthesis rate with functional regression, as implemented in the REFUND package (Goldsmith et al., 2016) in the R statistical environment (R Core Team, 2016). REFUND is particularly efficient for functional regression, as it employs wrapper functions that automatically fit various functional forms to predictors using a generalized additive mixed-model approach (Breslow & Clayton, 1993; Lin & Zhang, 1999). We specifically used the penalized functional regression function (pfr; Randolph et al., 2012) to characterize gradient functions with penalized splines.

The analysis of selection on lower sepal length considered a plant's total fruit set as the dependent variable and used a linear function predictor (If, Goldsmith et al., 2011) to fit psplines characterizing trait variation with respect to relative flower position. Total plant fruit production was modeled as a Poisson distribution with a ln link function. The analysis also included the logarithm of total flower number as a covariate for explaining fruit set variation.

The analysis of phenotypic selection on anthesis rate variation used a slightly different approach. In this case, we used total seed number as the dependent variable and p-splines to characterize the relation of anthesis rate to inflorescence age. However, in this case the functional relations could vary, depending on the maximum age at which the last flower opened on inflorescences (i.e., the domain of variation). To accommodate this variation, we used a linear variable-domain function (lf.vd) to fit thin-plate splines with second-order penalization (Gellar et al., 2014).

4 Results

For the sampled plants, we observed similar patterns of morphological and phenological variation within inflorescences to that reported previously for another Delphinium glaucum population (Gallwey, 2011; Ishii & Harder, 2012). Mean lower sepal length of D. glaucum flowers decreased with flower position from the bottom to the top of inflorescences $(F_{1,109,2} = 172.4, \text{ slope} \pm \text{SE} = -0.291 \pm 0.022 \text{ mm/relative posi-}$ tion: Fig. 1A). In addition, the daily rate at which new flowers opened (anthesis rate) declined as inflorescences aged $(F_{1.800} = 67.48, P < 0.001;$ partial regression coefficient for $\ln[\text{inflorescence age}] = -0.296 \pm 0.036$: Fig. 1B), after accounting for a positive effect of ln(total flower number) $(F_{1.800} = 177.49,$ P < 0.001; partial regression coefficient = 0.735 \pm 0.055).

Functional regression revealed significant phenotypic selection on the within-inflorescence gradient of sepal-length variation, as represented by penalized splines ($X^2 = 23.14, 3.3$ d.f., P < 0.001: Fig. 2). Plants with longer lower sepals on flowers at the base (positions 1-2) and top (positions 6-7) of the inflorescence produced more fruits than those with shorter sepals at these positions (i.e., positive regression coefficient). In contrast, the opposite pattern was apparent for middle flowers (positions 3–4), as plants with longer sepals in these flowers set fewer fruit. In addition, fruit production varied significantly with ln(total flower number) (partial coefficient \pm SE = 1.238 \pm 0.084, regression Z = 14.76, P < 0.001). The adjusted R^2 indicates that the fitted model accounted for 72.3% of the overall among-plant variation in fruit production.

Functional regression also detected significant phenotypic selection on within-inflorescence variation in anthesis rate, although the pattern of selection varied with the total duration over which plants opened new flowers ($X^2 = 120.77$, 53.2 *d.f.*, P < 0.001: Fig. 3). Regardless of the duration of the anthesis period, plants that initially opened flowers quickly produced more seeds than those that opened flowers slowly (i.e., positive regression coefficients). Selection generally weakened as plants opened flowers during four or fewer flowered) plants that opened flowers during four or fewer days selection became negative. However, for plants that opened flowers over six or more days selection again became significantly positive as plants opened their last flowers (Fig. 3B). Overall, the fitted model explained about 71.5% of the among-plant variation in seed production.



Fig. 1. Variation of least-squares mean (\pm SE) **A**, lower sepal length and **B**, anthesis rate (number of new flowers opened per day) within racemes of *Delphinium glaucum*. Curves represent the fits of generalized linear models. In **A**, relative flower position identifies the seventh of the inflorescence represented by sampled flowers, ranging from the bottom to the top of inflorescences. In **B**, inflorescence age identifies the number of days since the first flower opened on an inflorescence.

Based on estimates from 10 458 variable SNPs, genetic relatedness varied extensively among the 110 individuals sampled during 2014 (coefficient of genetic relatedness: 0.027–0.789). About 25% of the among-plant variation in regression coefficients describing within-inflorescence variation in anthesis rate was associated with genetic relatedness. Specifically, the narrow-sense heritability (\pm SE) for within-inflorescence anthesis-rate gradient was 0.245 \pm 0.119 (P < 0.05). In combination with the observed phenotypic



Fig. 2. Estimated gradient of phenotypic selection $(\pm 95\%)$ confidence interval) on lower sepal length within inflorescences of *Delphinium glaucum*. Relative flower position identifies the seventh of the inflorescence represented by sampled flowers, ranging from the bottom to the top of inflorescences.

selection, this result demonstrates significant natural selection on the pattern of change in anthesis rate within *D. glaucum* inflorescences.

5 Discussion

The preceding results demonstrate two approaches to the analysis of systematic gradients of within-inflorescence variation as function-valued traits: parameters as data, and functional regression. The parameters-as-data approach allowed assessment of genetic variation of within-individual variation. Similarly, functional regression permitted quantification of the effects of within-inflorescence gradients on overall plant performance, and hence the characteristics of phenotypic selection acting on architectural effects. These approaches revealed patterns that are recalcitrant or inaccessible with traditional statistical methods. For example, the striking evidence of variation in selection on lower sepal length within inflorescences (Fig. 2) could not have been exposed without functional regression. Further, functional data methods are necessary to characterize how selection on anthesis rate depends on the number of days over which plants opened new flowers (Fig. 3). Thus, recognizing that systematic gradients of floral-trait variation represent function-valued traits enables unique, direct analysis of the genetic determination, functional significance, and evolutionary consequences of architectural effects.

At least for lower-sepal length and anthesis rate, phenotypic selection did not act equally along *Delphinium glaucum* inflorescences, but rather varied with flower position and inflorescence age. Functional regression indicated selection favoring longer lower sepals on lower and upper flowers, whereas selection favored shorter sepals on middle flowers (Fig. 2). Similarly, inflorescences experienced strong selection for faster initial anthesis, followed by weaker or even negative selection, a pattern that was particularly evident for plants that opened flowers during a relatively brief period (e.g., <5



Fig. 3. A, Estimated gradients of phenotypic selection on anthesis rate and **B**, associated *Z* values for *Delphinium glaucum* plants that opened new flowers over periods of indicated durations. In panel **A**, the ordinate range has been limited to facilitate presentation of variation in regression coefficients for the majority of plants, which opened flowers over more than 4 days, truncating the illustrated range of variation in the estimated regression coefficient for plants that opened flowers for fewer than 5 days. In panel **B**, *Z* values more extreme than the horizontal dashed lines (\pm 1.96) identify statistically significant selection.

days: Fig. 3A). Such heterogeneous selection along inflorescences likely reflects the various aspects of pollinator interaction (Ohashi & Yahara, 2001; Ishii & Harder, 2012) and mating-system consequences (Harder & Barrett, 1995; Harder et al., 2004) associated with variation in flower and display size. For example, opening more flowers per day when inflorescences are young than during peak display may maintain sufficient floral displays for pollinator attraction while simultaneously limiting the negative effects of geitonogamy (Harder et al., 2004; Harder & Johnson, 2005; Gallwey, 2011). Nevertheless, the occurrence of phenotypic selection and its heterogeneity with respect to flower position indicate that the prevailing gradients of within-individual variation (Fig. 1) were not optimal; at least during the year that sampling occurred. For example, selection favored an even stronger gradient of lower sepal length for lower and middle

flowers, but a weaker gradient for upper flowers (Fig. 2). Whether this incongruity reveals recent change in the pollination and mating environment and/or the influence of functional or development constraint is not apparent from our analysis. As this example illustrates, the conclusion that selection can vary within inflorescences suggests new questions regarding the origin and function of this variation. In particular, to what extent do such patterns of selection shape variation caused by architectural effects?

Functional data approaches allow several advantages for the analysis of systematic within-plant variation. The utility of penalized spline fitting allows complete depiction of withininflorescence variation without *a priori* knowledge of the pattern of variation (Goldsmith et al., 2011). In addition, spline fitting and functional-trait smoothing techniques can accommodate missing observations (Reiss & Ogden, 2007) that otherwise decrease the power of statistical inference. Further, individuals within a population rarely produce the same number of repeated units (e.g., flowers) that comprise functional inflorescence traits. Variable-domain functional regression accounts for heterogeneous predictor sizes among inflorescences and importantly can capture how patterns of variation change over different domain sizes (Gellar et al., 2014).

The parameter-as-data approach used to characterize variation and estimate heritability of anthesis rate is suitable when gradients of variation can be described through parametric regression. However, when parametric regression does not adequately describe variation within all individuals, functional data analysis approaches are required. For example, the estimation of eigenvalues and eigenfunctions, that are akin to traditional principal components analysis, may be required to predict changes in the additive genetic covariance matrix (Kingsolver et al., 2001; Stinchcombe et al., 2012). These eigenfunctions highlight changes in functional traits that are proportional to the underlying genetic variation, or a lack of evolutionary capacity when genetic variation is limited. Alternatively, deviations of models for individuals from an overall population mixed model can be used to estimate the genetic covariance matrix (Rice & Wu, 2001; reviewed in Stinchcombe et al., 2012).

Functional data analysis techniques perform well with many data types that suffer from typical issues associated with biological studies (e.g., missing data), but their application still requires careful, thorough data collection. For example, although measurement of seven flowers per plant allowed assessment of the effect of relative flower position on selection acting on lower sepal length, this sampling was too sparse to apply the variable-domain method, given the total number of flowers per inflorescence. Although we included total flower number as a scalar covariate to account for its overall effect on selection, this approach does not allow assessment of the effect of flower production on the pattern of selection within inflorescences. In general, the ability of spline fitting to characterize within-individual variation improves with the density of sampling within individuals (regularization: see Morris, 2015). Finally, as with all parametric statistical methods, confidence in parameter estimation improves as samples include more individuals (Morris, 2015). Therefore, whereas functional data analysis techniques are generally robust to variable sampling designs, overall performance and robustness improve with more complete representation of within-inflorescence variation and larger samples.

Additional functional data methods not employed in this study could also be relevant to the analysis of inflorescence characteristics and performance. The functional regression technique applied here to characterize phenotypic selection on inflorescences used functional predictors (splines of lower sepal length, anthesis rate) and a single (scalar) response (fruit or seed set): scalar-on-function regression. Methods are also available to evaluate the relation of a functional response to one or more functional predictors, or a functional response to scalar independent variables (Ramsay & Silverman, 2005; Morris, 2015; Goldsmith et al., 2016). For example, these methods could respectively be used to assess positionspecific variation in performance (e.g., per-flower seed number) with either functional predictors that represent architectural effects (e.g., position-dependent variation in traits of individual flowers) or whole-plant characteristics (e.g., number of potential mates within a circumscribed area). Finally, functional ANOVA provides a means of testing whether mean functional responses differ among classes of categorical independent variables, such as species or experimental treatments (Barry, 1996; Cuevas et al., 2004).

Functional data techniques enable analysis of systematic gradients of within-plant variation. Despite increased availability of these techniques (Morris, 2015) and their application in other areas of biology (Stinchcombe et al., 2012), these methods have yet to be applied to gradients of morphological and phenological variation in plants, including architectural effects. We therefore advocate the use of both the parameters-as-data and functional regression approaches, as appropriate, to address existing and novel hypotheses concerning the function and evolution of inflorescence characteristics.

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