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Phenotypic integration and response to stress in *Arabidopsis thaliana*: a path analytical approach

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ABSTRACT

Questions: Does the pattern of phenotypic integration (correlations among characters) change with the environment? Do correlations become tighter with increased perceived stress? Is path analysis a useful analytical method to study phenotypic integration?

Organism: Several populations of the annual weedy plant *Arabidopsis thaliana*, a model system in both molecular and organismal biology.

Methods: Plants grown under four experimentally induced conditions, three of which were meant to impose increasing stress by manipulating water and light levels. The experiment was conducted in growth chambers, and the data were subjected to analyses of variance and path analyses.

Results: Plants did experience various amounts of stress, though not always in the predicted rank order. The patterns of phenotypic integration were more stable than expected, despite some environmental sensitivity of several path coefficients.

Conclusion: Path analytical models based on previous experience with the experimental system can be used to explore and quantify patterns of variation in character correlations.

Keywords: *Arabidopsis*, environmental stress, path analysis, phenotypic integration, phenotypic plasticity.

INTRODUCTION

Phenotypic integration is a loosely defined term that has been applied to a variety of approaches and problems in organismal biology, from Olson and Miller (1958) to Pigliucci and Preston (2004). Broadly speaking, research on phenotypic integration poses questions related to how (genetically and developmentally) and why (in terms of adaptation and natural selection) suites of characters are or are not correlated (integrated) with each other.

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As such, the study of integration spans the whole of organismal biology, with questions and methods borrowed from genetics, molecular biology, developmental biology, ecology, and evolutionary biology. In practice, of course, any given study of the degree of integration (or lack thereof) of particular phenotypic traits has to focus on a subset of questions and corresponding methods. Here we concentrate on two specific questions that address the patterns of among-population integration and environmentally induced changes (phenotypic plasticity) of character correlations.

Much comparative evolutionary biology research is carried out either within populations or across species. The first level of analysis addresses questions related to the very recent history of phenotypic evolution, as well as to the immediate potential of populations to respond to short-term selective pressures. The latter level of study examines the relatively long-term outcome of evolutionary processes, in so far as they are reflected in the patterns of species diversity within a phylogenetic context. There is, of course, an intermediate level of analysis, which – though more rarely used – provides a conceptual bridge between the two mentioned above: this is the study of phenotypic variation when populations within a given species are the data units [for a discussion of the importance of this level of evolutionary analysis, see Armbruster *et al.* (1996)]. Similar to the situation holding for the classic literature on inter-population allometry (Gould, 1966), we are here looking at the effects of relatively recent evolutionary processes (selection, migration, etc.), as well as gaining a glimpse of the potential for further differentiation, possibly leading to speciation [by ecological or other means (Hoffman and Blows, 1994)]. In a sense, then, population-level studies can be thought of as providing a bridge between micro- (within populations) and macro- (across species) evolution.

Another aspect of integration that is assessed in this paper concerns the phenotypic plasticity of character correlations. This phenomenon has been highlighted and discussed by Schlichting (1986, 1989), and has received some attention both from a theoretical (e.g. Stearns, 1991; Diggle, 2002) and empirical standpoint (e.g. Lechowicz and Blais, 1988; Newman, 1994; van Tienderen and von Hinsberg, 1996; Pigliucci and Kolodynska, 2002a, 2002b). There are two main issues underlying such interest. The first is the extent to which trade-offs between different phenotypic characters may be altered by environmental changes (e.g. Leroi *et al.*, 1994; Sinervo and Svennson, 1998; Roff and Gelinas, 2003). The second is the likelihood that specific suites of correlated traits reflect the concerted evolution of those traits as favoured by natural selection [adaptive multivariate plasticity (e.g. Losos *et al.*, 2000; Donohue *et al.*, 2001; Bouton *et al.*, 2002)].

In this paper, we use a set of natural populations of the weedy annual *Arabidopsis thaliana* (Brassicaceae) to investigate both the pattern of phenotypic integration at the across-population level of analysis, and whether and how such a pattern is altered by changes in important aspects of the environment. *Arabidopsis thaliana* is a well-established model system in molecular and developmental biology (Anderson and Roberts, 1998), and has increasingly been used in organismal biology and as a link between reductionist and whole-organism approaches (e.g. Pigliucci, 1998; Mitchell-Olds, 2001; Jackson *et al.*, 2002). We used a path analytical approach – relatively common in studies of phenotypic integration (e.g. Farris and Lechowicz, 1990; Pigliucci and Schlichting, 1998; Scheiner, 2000) – to test a model of causal interactions among morphological and life-history traits (Shipley, 2000), derived from a combination of previous path analytical studies of this species and our own long experience with this model system. We then explored how the paths so identified were affected by increasing degrees of two environmental stressors: water availability (from 'standard' to flooded conditions) and light (from 'standard' to low levels).

Specifically, we asked the following questions:

1. To what extent does previous knowledge of the developmental ecology of *Arabidopsis* allow one to build models of causal interactions that are consistent with observed variance–covariance matrices among measured traits?
2. Do environmental conditions intended to place the plants under significant stress really cause a measurable decrease in reproductive fitness, and do they do so in the rank order predicted by the investigators on the basis of our understanding of the plant's autecology?
3. Do path coefficients describing phenotypic integration in *Arabidopsis* change the magnitude and/or sign depending on the imposed environmental conditions?
4. If the latter is the case, do more stressful environments elicit more phenotypic integration (i.e. a higher number of strong inter-trait coefficients), do they have the opposite effect (i.e. 'loosening' up the phenotype, so to speak), or is there no consistent relationship between stress and integration?

MATERIALS AND METHODS

We used a sample of 21 wild accessions representing early flowering populations of *Arabidopsis thaliana* from Europe (the major natural area of distribution of the species) available from *The Arabidopsis Information Resource* (TAIR; available at www.arabidopsis.org). These lines are representative of natural populations of *A. thaliana* collected in the field, and are maintained under uniform conditions at the stock centre, to be shared by researchers interested in different aspects of *A. thaliana*'s biology. The lines we used were: CS911 from Estland (Germany); CS913, ecotype RLD from an unidentified location in the Netherlands; CS922 from Hodja-Obi-Garm (Tadjikistan); CS925 from Litva (Lithuania); CS932 from Kingswells (Aberdeen, Scotland); CS1214 from Glueckingen (Germany); CS1226 from Hilversum (Netherlands); CS1252 from Vranov Brna (Czech Republic); CS1282 from Koeln (Germany); CS1604 from Wietze (Germany); CS1630 from Wildbad (Germany); CS1635 from Canterbury (England); CS3179 from Graz (Austria); CS6023 from Sidmouth (Devon, England); CS6034 from Bretagny sur Orge (France); CS6041 from Kelsterbach (Germany); CS6046 from Koeln (Germany); CS6068 from Kent (England); CS6194 from Blanes (Spain); CS6195 from Wu (Germany); and CS6731 from Glueckingen (Germany).

To minimize maternal effects and increase seed availability, we grew the material for one generation under controlled laboratory conditions (a 16 : 8 h light : dark cycle, at a room temperature of 23–25°C, using standard pro-mix soil, with bottom watering provided every other day to reduce mechanical interference, while maintaining the plants under good growth conditions). Second-generation seeds were then placed on a wet filter paper and cold-treated for 1 week at 5°C in a refrigerator to enhance and synchronize germination. Imbibed seeds were transferred to soil and placed under combined fluorescent and incandescent lights on growth racks. Seedlings were randomly thinned after 5 days, when the appearance of the first true leaves was noted, leaving one plant per pot (7 cm diameter × 5 cm deep).

We applied four treatments, two levels of light availability and two of water, combined in a full factorial experimental design:

- 'standard' water and 'standard' light (the term 'standard' here refers to typical growing conditions for *A. thaliana* in growth chambers: for water, this means that the soil was always wet but not saturated; for light, the plants were experiencing an average of 240 $\mu M \cdot m^{-2} \cdot s^{-1}$ photon flux);
- standard water and low light (i.e. 70 $\mu M \cdot m^{-2} \cdot s^{-1}$ photon flux);
- flood-level water (i.e. pots always saturated) and standard light; and
- flood-level water and low light.

Plants assigned to standard water treatments were bottom watered every other day, letting the soil saturate with water for 2 h and then drained. The flooded treatments had the water changed every other day (at the time when the control group was watered) to minimize algal growth. Our prediction was that the plants would experience a stress gradient (measured by a progressive reduction in reproductive output; see below), with the following sequence (from least to most stressful): standard/standard > standard water/low light = flood/standard light > flood/low light. The flood-water conditions represent a rather novel environment for this species, since it is not usually experienced in the field; on the other hand, low light – while still presumably stressful – is not rare for natural populations of *A. thaliana* (M. Pigliucci, personal observation). The flooded treatment therefore was used to test the hypothesis that ecologically novel environments are likely to elicit stress, because populations have not been exposed to such environments during their recent evolution, and have presumably not evolved adaptive responses to them. This in turn makes novel or stressful environments suitable to explore how patterns of phenotypic integration may be altered by stress (see questions in the Introduction).

We began the experiment with 21 accessions, four treatments, and six replicates per accession/treatment combination (with the replicates randomly assigned to two growth chambers), for a total of 504 individuals. We experienced low random levels of mortality, which reduced the final sample size to 441 individual plants. We measured six standard traits, often used in morphometric studies of *A. thaliana* (e.g. Pigliucci and Kolodynska, 2003): (1) number of leaves in the basal rosette (a measure of meristem allocation during the vegetative growth); (2) mature rosette diameter (at flowering, a measure of vegetative growth); (3) bolting date (the time when the plants switch from vegetative to reproductive phases); (4) shoot weight (a measure of allocation to reproductive structures); (5) time to senescence (defined as the drying up of all aerial structures, especially reproductive ones); and (6) number of fruits produced (a proxy for reproductive fitness, given that this is an annual plant).

As a preliminary step to the structural equation modelling of the data, we ran standard analyses of variance (ANOVA) on each trait. Growth chamber effects were never statistically significant, so they were dropped from the final model. The growth chamber effect, the population effect, as well as all the latter's interactions, were considered random, while all other effects were treated as fixed in the ANOVA models. We had to transform data for every measured character, because of deviations from either the assumption of normality or that of homoscedasticity. The transformations were chosen so as to reduce or eliminate these problems, following the suggestions in Sokal and Rohlf (1995), and resulted in using the square root of leaf number and the logarithms of all other traits. We also standardized the data (centring on the mean, dividing by the standard deviation) before both the analyses of variance and structural equation modelling to make all outputs directly comparable among traits for the univariate analyses. Univariate analyses were carried out using Jump software for Mac. Contrary to usual practice, we do not report the results of

Bonferroni-like corrections for multiple tests, for the excellent reasons raised against such usage by Moran (2003). Instead, we report the 'native' *P*-values and discuss the results in a biologically and statistically reasonable fashion, leaving to the informed reader the assessment of the overall value of our findings.

Structural equation modelling (path analysis) is a very flexible technique that allows testing of specific causal hypotheses based on how well they predict an observed covariance structure among a given set of variables (Shipley, 2000). Even for a relatively small number of variables, it is usually not feasible to test all available models and choose the one that fits the data best. There are two reasons for this. First, many models do not make any biological sense (e.g. they assume a reversal of the known causal flow during the development of the organism). Second, several alternative models can equally (or almost equally) 'fit' the data according to a variety of statistics, and an exercise in simple model fitting is hardly biologically informative. Instead, one typically explores the properties of one or a small set of biologically reasonable models (arrived at from previous knowledge of the system at hand), using the results to gain insights into which further directions of research to pursue.

We carried out our structural equation modelling using the LISREL software for Mac (Joreskog and Sorbom, 1996), using both the SIMPLIS and the LISREL output options, which provide a variety of hypothesis testing tools and summary statistics. We began by considering a series of models already proposed in the literature for how morphological and life-history traits relate to each other and to measures of reproductive fitness in *Arabidopsis thaliana*: (1) a model developed by Pigliucci *et al.* (1995) to include seven traits plus fruit production; (2) a model proposed by Pigliucci and Schlichting (1998) based on four traits and fruit production; (3) a path diagram published by Scheiner *et al.* (2000) with four traits and fruit production; and (4) a model by Scheiner *et al.* (2002) with six traits and fruit production. We combined the common elements of these models and adapted them to the particular set of traits we had measured, based in part on our own long experience with growing *A. thaliana* under a variety of environmental conditions. The resulting causal model and path diagram are illustrated in Fig. 1. The number of leaves and diameter of the rosette (relatively early ontogenetic traits) are considered to be intercorrelated for reasons that are outside the scope of the model (presumably at least partly because of ontogenetically even earlier traits); they both have direct effects on three later characters: bolting date, time to senescence, and shoot weight. Following an ontogenetic cascade, bolting date is then

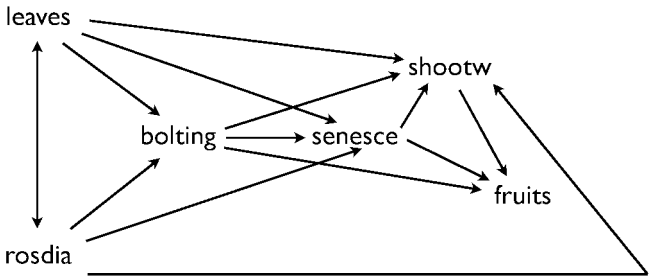

Fig. 1. The path analytical model used to fit the data in all four treatments. The model is based on previously published path analyses of similar traits in *Arabidopsis thaliana*, as well as on the authors' experience with the phenotype and ontogeny of this species. Leaves = number of leaves, rosdia = diameter of rosette, bolting = bolting date, shootw = shoot weight, senesce = time to senescence, fruits = number of fruits.

expected to affect time to senescence, shoot weight, and number of fruits (our measure of reproductive fitness); time to senescence is expected to affect shoot weight and number of fruits; and shoot weight is expected to affect number of fruits. LISREL then calculated the indirect effects embedded in such a causal model.

The basic model was then applied to four subsets of the data, one for each combination of treatments to which we exposed the plants. In each case, model performance was evaluated by a variety of criteria based on the LISREL output. We focused on and report the following:

1. *Root mean square error of approximation* (RMSEA), together with its confidence interval and *P*-value for the hypothesis of a RMSEA = 0. The RMSEA assesses a given model's fit to the observed covariance matrix while accounting for the number of parameters embedded in the model itself. This is useful because the more parameters that are present, the more a given model will fit the data, other things being equal. The RMSEA is more appropriate than the often used chi-square statistic because the latter does not take into account the number of parameters in the model.
2. *Akaike's information criterion* (AIC), calculated for three models: the one actually being tested, as well as a saturated model and an independence model. The saturated model has no degrees of freedom, the path diagram is similar to that of the model being tested, but it includes all possible paths, yielding an over-parameterized model with the maximum possible fit. The independence model has no constraints – that is, all variables are actually assumed to be independent of each other (this is essentially equivalent to the simple path diagram generated by a multiple regression analysis). If the tested model captures the causal structure sufficiently well, its AIC is expected to be close to that of the saturated model and away from that of the independence model.
3. Finally, we also examined the *goodness-of-fit index* for the model in each environmental circumstance; this index varies from 0 (complete lack of fit) to 1 (perfect fit).

Besides considering the overall statistics of fit of the tested model for each environmental setting, we plotted the reaction norms of the path coefficients representing both the total and indirect effects described by the model, together with their 90% confidence interval. Our aim was to examine the environmental dependence of the various components of the hypothesized causal model.

RESULTS

Before examining the main focus of our analyses, the results of the structural equation modelling approach, we briefly outline the output of standard analyses of variance carried out on each trait. The full model included three main effects, three two-way interactions, and one three-way interaction (other than the error term). The amount of phenotypic variance explained for each trait ranged from a minimum of 51% (time to senescence) to a maximum of 73% (number of leaves) (Table 1).

Since trait values were standardized before the analyses of variance were carried out, the mean squares for each trait–factor combination are directly comparable across traits, which helps when interpreting the relative importance of each factor in the analysis in explaining the phenotypic variation of any given trait. It is interesting to note that for almost all characters, the greatest part of the phenotypic variance was associated not with the genetic

Table 1. Analyses of variance for the six traits (transformed and standardized data, see text). Numbers in parentheses below effect type are degrees of freedom. Numbers in table are Mean Squares, with *P*-values of the corresponding *F*-tests in parentheses. R^2 is the amount of variance explained by the model. Population and its interactions were considered random effects. Microenvironmental effects were not significant and excluded from the final analysis. For Light and Water effects, we report the relative rank of each trait's means (e.g. low > stand means that the trait mean was higher under low light than under 'standard' conditions); for the Light by Water interaction, ranks of the four treatments (L = low light; F = flooded; S = standard light or water) are reported for the three traits for which the effect was statistically significant.

Trait	Population (20)	Light (1)	Water (1)	Pop × Light (20)	Pop × Water (20)	Light × Water (1)	Pop × Light × Water (20)	Error (299)	R^2
Leaf no. (sqrt)	9.26 (0.0005)	11.29 (0.0023) low > stand	11.33 (< 0.0001) stand > flood	0.96 (0.0725)	0.22 (0.9586)	0.91 (0.1844)	0.49 (0.0401)	0.30	73%
Rosette diameter (log)	2.16 (0.0230)	62.79 (< 0.0001) low > stand	52.81 (< 0.0001) stand > flood	0.56 (0.3003)	0.37 (0.6583)	3.16 (0.0143) LS > LF = SS > SF	0.44 (0.5825)	0.49	58%
Shoot weight (log)	1.05 (0.1656)	34.11 (< 0.0001) low > stand	114.19 (< 0.0001) stand > flood	0.53 (0.4734)	0.30 (0.8803)	9.27 (0.0003) LS > SS > LF > SF	0.51 (0.3215)	0.45	65%
Bolting (log)	7.32 (0.0008)	37.17 (< 0.0001) low > stand	1.87 (0.0118) flood > stand	0.35 (0.4372)	0.24 (0.7375)	0.26 (0.3849)	0.33 (0.4326)	0.32	69%
Senescence (log)	2.12 (0.1400)	61.46 (< 0.0001) low > stand	1.73 (0.1437) flood = stand	1.06 (0.1165)	0.76 (0.3225)	0.52 (0.3672)	0.61 (0.3800)	0.57	51%
Fruits (log)	0.94 (0.6039)	17.07 (< 0.0001) low > stand	122.83 (< 0.0001) stand > flood	0.65 (0.7578)	0.38 (0.9664)	9.11 (0.0040) LS = SS > LF > SF	0.89 (0.0124)	0.47	63%

differentiation among populations, but with the main effect of light and/or water treatments. Conversely, the interaction effects explained little phenotypic variance, with the partial exception of the light × water factor (Table 1). This means that environmentally induced phenotypic variation was more important than either the overall genetic differentiation or the genetic differentiation for plasticity in the populations and environmental settings examined.

If we consider the main environmental effects in more detail, we see that plants grown under low light (versus standard light) were characterized by more and larger leaves, more allocation to shoot weight (perhaps surprisingly), later flowering time, later senescence, and a higher reproductive output (again, rather surprisingly). Plants grown under the flooded regime had fewer and smaller leaves, produced smaller inflorescences, but also flowered later; they did not show a difference in time to senescence compared with the standard water conditions, but they ended up producing fewer fruits (Table 1). The details of the light × water interaction were also interesting: no overall difference was found for leaf production, but rosette diameter was largest under low light/standard water, smallest under standard light/flood, and intermediate in the other two combinations of environments. Shoot weight followed a very similar pattern, with the exception of a detectable difference between the two intermediate environments (heavier inflorescences in standard light/standard water than in low light/flood). Interestingly, no overall differences among the four environments were observed for the two life-history traits (bolting date and time to senescence) (Table 1).

The plants' behaviour across environments for fruit production was especially important, since this was our way of assessing the actual stress impact of the four treatments. Contrary to our expectations (see Materials and methods), the most stressful environment was standard light/flood, although the other three combinations of treatments did rank according to our predictions, with standard light/standard water eliciting the highest reproductive output (Fig. 2). Note that this anomalous result was probably a consequence of relatively extensive algal growth under the standard light/flood conditions, with the algae

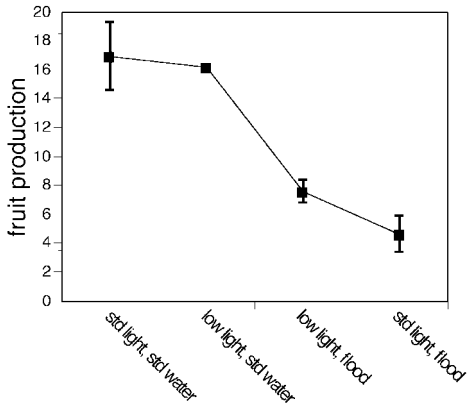

Fig. 2. The ranking of the four environments in terms of perceived stress by the plants, as measured by their overall fruit production. Note that contrary to predictions, the most stressful environment was standard light/flood rather than low light/flood. This was probably because the combination of a higher availability of light and water stimulated significant algal growth. Even though we constantly scraped the algae from the surface of the pots, its presence probably had a negative effect on the fitness of *Arabidopsis*. Bars indicate 90% confidence intervals.

taking advantage of the relatively high light levels, despite our precautions to avoid just such a problem. Algal growth is notorious for having a negative effect on *Arabidopsis* growth and fruit production. This unintended, but rather interesting, biotic effect intervened to complicate our experimental set-up. In the following, we present and discuss the results of the structural equation modelling using the actual, not the predicted, stress-rank of the four treatments.

We used the structural equation model arrived at as explained earlier (Fig. 1) to fit each of the four environment-specific data sets to construct reaction norms for the total and indirect path coefficients (the direct effects can be obtained by subtracting the two). For the sake of clarity, we examine the total and indirect effects in a sequence parallel to the ontogeny of the plant, beginning with the effects of early traits such as leaf number and size, and finishing with the effects of late traits such as time to senescence and inflorescence weight.

The two-directional path between leaf number and rosette diameter (Fig. 3) was always positive and well above zero in all treatments, though slightly higher under low light/standard water (a low-stress treatment). Leaf number also had a consistently positive overall effect on bolting date, as is usually the case in *A. thaliana* populations; interestingly, the other total effects of leaf number (on senescence, shoot weight and fruit production) were close to zero in the two standard light environments, but negative in both low light conditions (i.e. under low light, more leaves translated into earlier senescing, smaller inflorescences and fewer fruits, regardless of water availability). According to our model, leaf number had three indirect effects, on senescence, shoot weight, and fruit production (Fig. 4): the path coefficients to shoot weight were essentially always zero, regardless of treatment; on the other hand, more leaves indirectly meant later senescence and fewer fruits under the two low light conditions, but made little or no difference in the two standard light environments.

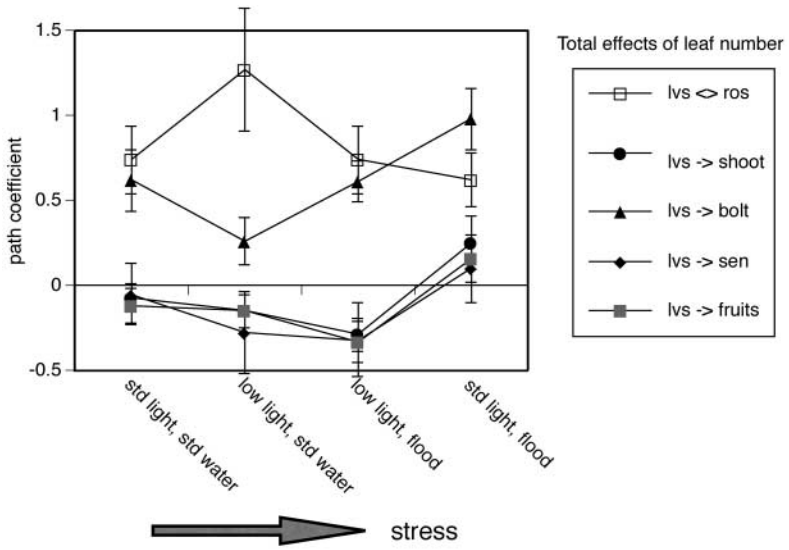

Fig. 3. Reaction norms of the total effects of path coefficients between the ontogenetically early trait 'number of leaves' and other characters. Bars indicate twice the standard errors associated with each path coefficient. lvs = number of leaves, ros = diameter of rosette, bolt = bolting date, shoot = shoot weight; sen = time to senescence; fruits = number of fruits. Bars indicate 90% confidence intervals.

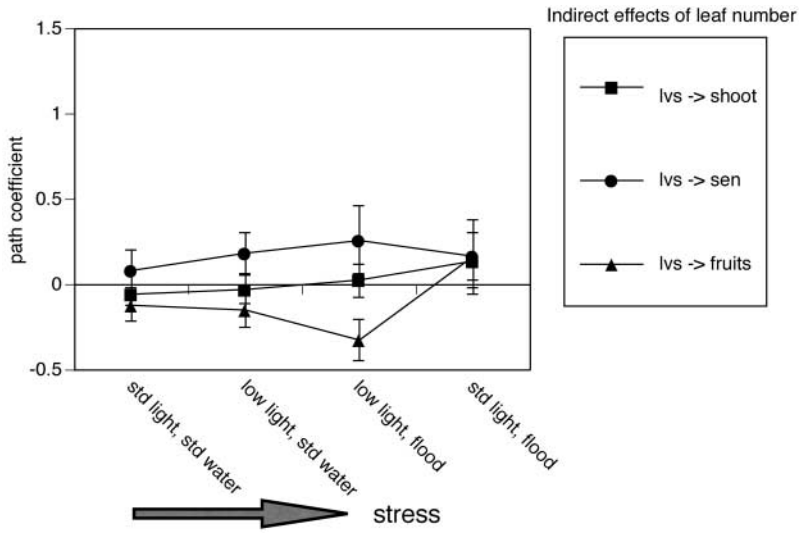

Fig. 4. Reaction norms of the indirect effects of the path coefficients between the ontogenetically early trait 'number of leaves' and other characters. Bars indicate twice the standard errors associated with each path coefficient. lvs = number of leaves, ros = diameter of rosette, bolt = bolting date, shoot = shoot weight; sen = time to senescence; fruits = number of fruits. Bars indicate 90% confidence intervals.

Considering the total effects of rosette diameter (Fig. 5), it is notable that larger values of this trait always translated into larger shoots and more fruits; there was scarcely an effect on time to senescence, while the relationship with bolting date was more complex and rather idiosyncratic (larger leaves translated into later bolting under low light and standard water, but earlier bolting under standard light and flooding, with little effect in the other two environments). The indirect effects of rosette diameter (Fig. 6) were more straightforward, with a constant across-environment positive effect on fruit production, no indirect effect at all on shoot weight, and largely no indirect effect on time to senescence (the exception being later senescence under low light/standard water).

For the total effect of ontogenetically later characters (Fig. 7), most of them were close to zero under all treatments, with some notable exceptions: larger shoots, not surprisingly, always translated into more fruits; and later bolting meant later senescing, but only in the two low light treatments. For the indirect effects of the same traits (Fig. 8), they were all constantly close to zero, with the exception of the low light/standard water treatment; however, although all indirect effects were positive here, the magnitude of the path coefficients was rather small.

DISCUSSION

Every living organism displays a degree of integration among its various phenotypic traits, from the molecular to the physiological to the morphological levels. The open questions are how (genetically as well as developmentally) such integration is achieved, and why (in terms of ecology and evolution) it occurs (see recent reviews and discussions of these and related topics in Pigliucci and Preston, 2004). Sinervo and Svensson (1998) discussed this distinction between mechanistic

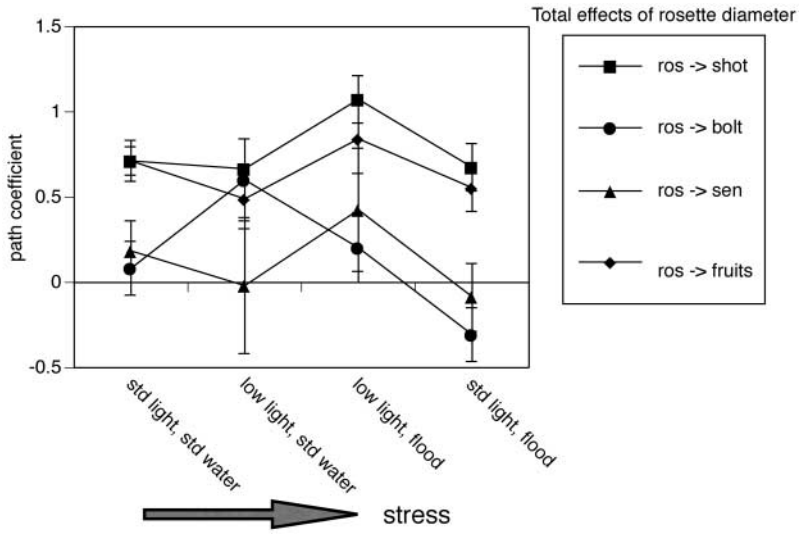

Fig. 5. Reaction norms of the total effects of path coefficients between the ontogenetically early trait 'diameter of rosette' and other characters. Bars indicate twice the standard errors associated with each path coefficient. lvs = number of leaves, ros = diameter of rosette, bolt = bolting date, shoot = shoot weight; sen = time to senescence; fruits = number of fruits. Bars indicate 90% confidence intervals.

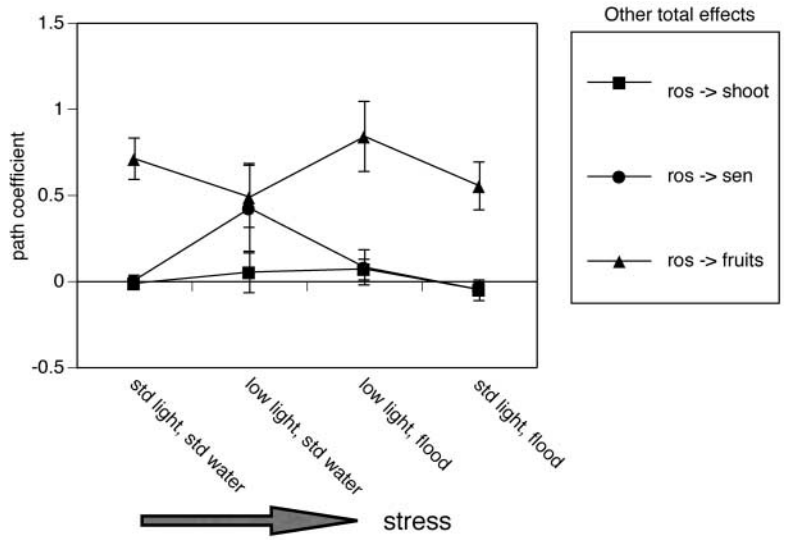

Fig. 6. Reaction norms of the indirect effects of the path coefficients between the ontogenetically early trait 'diameter of rosette' and other characters. Bars indicate twice the standard errors associated with each path coefficient. lvs = number of leaves, ros = diameter of rosette, bolt = bolting date, shoot = shoot weight; sen = time to senescence; fruits = number of fruits. Bars indicate 90% confidence intervals.

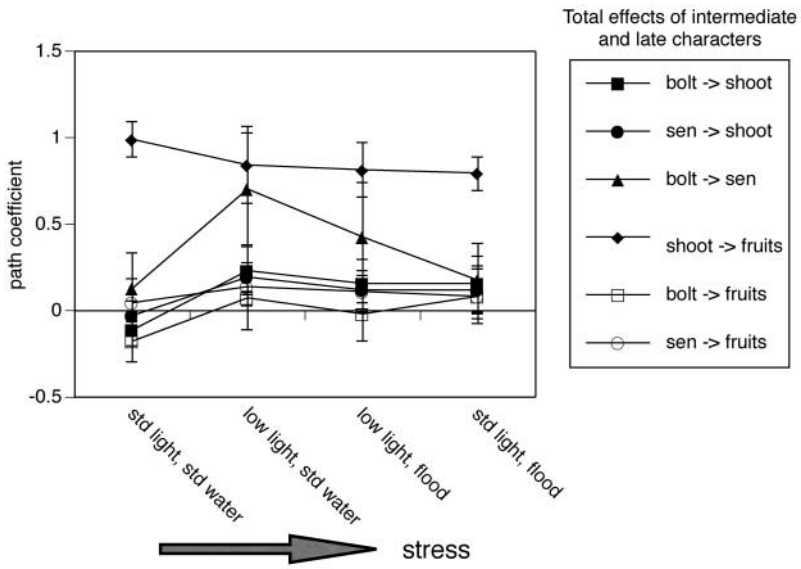

Fig. 7. Reaction norms of the total effects of path coefficients between the ontogenetically intermediate and late traits. Bars indicate twice the standard errors associated with each path coefficient. lvs = number of leaves, ros = diameter of rosette, bolt = bolting date, shoot = shoot weight; sen = time to senescence; fruits = number of fruits. Bars indicate 90% confidence intervals.

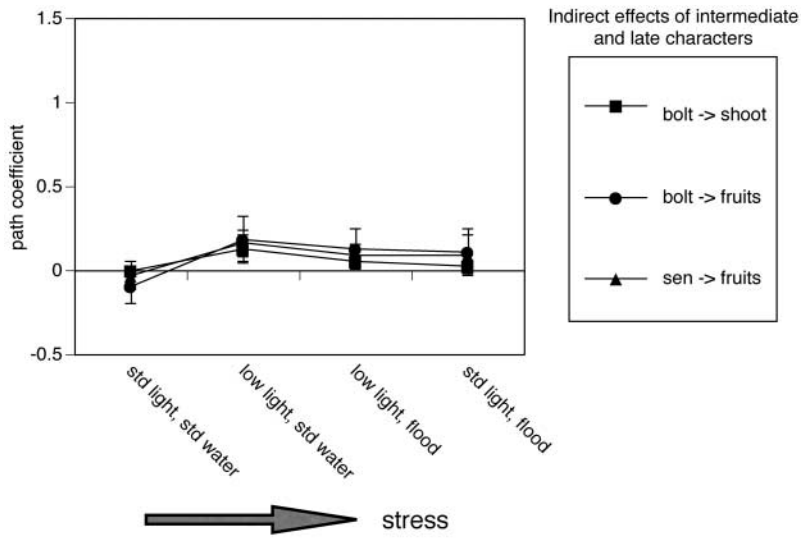

Fig. 8. Reaction norms of the indirect effects of the path coefficients between the ontogenetically intermediate and late traits. Bars indicate twice the standard errors associated with each path coefficient. lvs = number of leaves, ros = diameter of rosette, bolt = bolting date, shoot = shoot weight; sen = time to senescence; fruits = number of fruits. Bars indicate 90% confidence intervals.

(proximate) and selective (ultimate) causes, and how they are related to each other within the framework of evolutionary ecology (see also Pigliucci, 2003), and reject the argument made by others that experimental manipulations are only informative about phenotypic plasticity and do not tell us anything about the physiology underlying observable genetic and phenotypic correlations. These are important and broad questions, but science makes progress most often when focusing on more specific, sometimes apparently even trivial, aspects of the natural world. Hence the need to link the general study of phenotypic integration to actual research on individual organisms conducted in the field or the laboratory. We have chosen several populations of the model system *Arabidopsis thaliana* to address a set of questions spurred by our wider interest in the evolutionary ecology of phenotypic integration.

In particular, we wished to ascertain whether:

- previous knowledge of the life history and ontogeny of *A. thaliana* could suggest a suitable hierarchical model of causal interactions among phenotypic traits that would be able to account for observable inter-trait covariance matrices;
- intended environmental stresses, imposed because of existing information about the plant's ecology, would in fact decrease *A. thaliana*'s reproductive fitness in the manner expected;
- environmental changes, and in particular two forms of stress, would quantitatively alter such causal relationships;
- there is a consistent relationship between the degree of stress as perceived by the plant and the pattern of phenotypic integration as quantified by path analytical techniques.

A priori knowledge and the prediction of observed covariances

Several years of experience working with *A. thaliana*, both on our part and that of other researchers (e.g. Pigliucci *et al.*, 1995; Pigliucci and Schlichting, 1998; Scheiner *et al.*, 2000, 2002), made it feasible to build a causal model relating morphological and life-history traits in a way that would allow us to predict with reasonable accuracy the observed covariance matrices in a set of environmental conditions. Judging from a variety of indexes (Table 2), our model fared well under three environments, although significant deviations between the predicted and observed covariance matrices were found for data pertinent to the low light/standard water treatment. However, even in the latter case, both the Akaike information criterion and the goodness-of-fit index were consistent with a reasonably fit model. One caveat is that it is possible that an environmental change might alter not just the magnitude of a path coefficient within a given model, but in fact the very structure of the causal relationships among the observed variables, and hence the path model itself. We did not consider this possibility here for two reasons. First, there is no accepted framework for how to compare models with arbitrarily different structures, unless such models happen to be a subset of each other. Second, looking for entirely different structures in the different environments would at this point be a fishing expedition, resulting in a simple exercise in model fitting rather than hypothesis testing. Nonetheless, we are aware that this problem ought to be tackled by students interested in environmentally dependent effects on the causal pathways underlying phenotypic integration, and model organisms such as *A. thaliana* may be the most promising loci with which to begin this sort of work.

Table 2. Summary statistics for the path analyses within each treatment. For various models being compared, 'saturated' refers to a model with all paths allowable, which has no degrees of freedom and perfect fit; 'independence' refers to a model with no constraints between variables; 'model' is the partially constrained path analytical model actually being tested. The Root Mean Square Error of Approximation (together with its 90% confidence and *P*-value – **boldface** indicates significant deviation from zero) is one of the best measures of fit between the observed covariance matrix and the one predicted by the model; unlike the often used Chi-Square, RMSEA corrects for the number of parameters included in the model, and is therefore a better measure of genuine fit (where good fit is indicated by RMSEA values close to zero). The fit of the tested model is better in proportion to how much closer its AIC (Akaike Information Criterion) is to the AIC of the saturated model, and away from the AIC of the independence model. The goodness-of-fit index varies between 0 (worst fit) and 1 (best fit).

Treatment	RMSEA	Confidence interval for RMSEA (*P*-value)	Saturated AIC	Model AIC	Independence AIC	Goodness-of-fit index
Low light/flood	0.13	0.0–0.26 (0.10)	42.0	43.80	321.94	0.98
Low light/ standard water	0.29	0.18–0.42 **(0.00048)**	42.0	56.15	292.20	0.94
Standard light/ flood	0.14	0.029–0.27 (0.075)	42.0	44.55	425.80	0.98
Standard light/ standard water	0.0	0.0–0.18 (0.52)	42.0	39.69	459.72	1.00

The model we explored was constructed on the simple assumption that ontogenetically early traits will have a cascading effect on later traits, with direct effects (paths) only on traits not too far removed ontogenetically. Two life-history characters, bolting date and time to senescence, are central to the path diagram, in that they are affected by early vegetative traits (leaf number and size) and in turn affect later reproductive characteristics (shoot weight and fruit production). Of course, a shortcoming of this path analytical model was that the relationship between leaf number and size of the rosette was left unexplored, with the two variables simply connected by a correlation, not explicitly causal arrows. This is because we did not measure any phenotypic trait that was ontogenetically prior to these two vegetative characteristics. It is not a trivial task to identify and reliably measure such early traits, although candidates for future research may include time to germination, size of the seedling, growth rate during the vegetative phase, as well as relevant physiological traits such as photosynthetic efficiency and chlorophyll content.

Path analysis based on previous biological knowledge of a given system has been successfully used in the study of phenotypic integration in the past. For example, Svensson *et al.* (2002) investigated the complex relationships among condition, colour morph, social environment, and female survival in side-blotched lizards. Their experimental-manipulative approach clearly showed that levels of corticosterone have pleiotropic effects, both direct and indirect, on reproductive traits and on the animal's condition, thereby providing a clear physiological basis for the observed phenotypic integration among such traits. Callahan and

Waller (2000) employed path analysis to study integration in populations of the annual plant *Amphicarpaea bracteata*, and concluded that while there was significant inter-population variation in patterns of integration for performance traits, such variation was not associated with the ecological conditions typical of the locations of origin of the plants, perhaps suggesting genetic and/or developmental constraints as the more likely explanatory factors. In general, therefore, path analysis and structural equation modelling may play a major role in exploring our understanding of causal pathways connecting phenotypic traits and how they respond to environmental challenges, despite the well-known drawback that one cannot test all, or even the majority, of all possible models. That is why the use of path analysis is inherently coupled with an intimate knowledge of the biological system and with the objective of testing specific models, rather than as an exploratory technique.

Predictability of plants' response to stress

We imposed four treatments on our plants, three of which were intended to be stressful (either because of low light or too much water, or a combination of both). We predicted that one of these stresses (the low light/high water combination) would have a negative effect on the reproductive output of the plants in a particularly clear fashion. In reality, the plants perceived only two of the four environments to be significantly stressful, and not in the rank order we expected. First, the difference between 'standard' light (i.e. the light level typically used in *Arabidopsis* experiments) and low light – while keeping water constant at the standard level – did not translate into a biologically or statistically relevant fall in fruit production. This is somewhat surprising, given that *A. thaliana* is not a shade-adapted organism. On the other hand, it is possible that the photosynthetic efficiency of our populations was already low enough under 'standard' conditions (which, after all, was much lower than in full sunlight), that a further lowering of the photon flux did not make any measurable difference. In this sense, our control plants were probably already somewhat stressed. While this is certainly a reason to conduct similar experiments under more realistic conditions (field or greenhouse), the drop in reproductive output between these two and the remaining two treatments was sharp enough to be biologically interesting and to justify our treatment of the two flooded conditions as 'stressful'.

Second, while obviously flooded conditions were perceived to be stressful regardless of the light intensity, the drop in reproductive fitness was – rather unexpectedly – more dramatic for the 'high light/flood' combination of environmental treatments. This outcome can probably be explained by the fact that the higher level of light triggered a significant algal growth when the pots were very wet. Even though we kept removing the algae on a regular basis, their growth may still have been significant enough to have a negative effect on *A. thaliana*'s reproductive fitness. Note that this problem was observed by Pigliucci and Kolodynska (2003), which is why we attempted to take appropriate precautions in the present study; it would appear that such precautions were not sufficient to avoid the problem. Our results here highlight both the difficulty of making accurate predictions even at the level of relatively uncomplicated ecological situations, and the fact that only repeated observations of the growth conditions (e.g. the detection of algal growth) allows one to begin to explain otherwise puzzling results.

Plasticity of path coefficients

One of our objectives was to quantify the extent, if any, of possible alterations in the pattern of phenotypic integration (as measured by path coefficients) induced by the various treatments we imposed. Pigliucci *et al.* (1995) compared the response of genotypes of *A. thaliana* to several conditions (including low light and low water, administered separately) and found strong phenotypic plasticity for all the traits they analysed, including several components of reproductive fitness. Also, Pigliucci and Kolodynska (2002b) studied phenotypic integration in response to flood in the same accessions of *A. thaliana* used here, and were surprised to find that the overall pattern of phenotypic integration changed little between treatments, though they also uncovered evidence of possible trade-offs between allocation to roots and above-ground biomass, as well as between leaves and reproductive structures. An analogous, but equally surprising, stability of integration patterns to environmental heterogeneity was found by the same authors when responses to different levels of light availability were analysed in the same genotypes of *A. thaliana* (Pigliucci and Kolodynska, 2002a). Herrera *et al.* (2002) examined phenotypic integration in *Helleborus foetidus* and found that the observed patterns of floral integration were closer to those expected based on developmental rather than ecological (pollinator-mediated) considerations, which should caution against any straightforward ecological interpretation of character correlations [see also Armbruster and colleagues' (1999) only partial confirmation of Berg's (1960) original hypothesis about the relationship between phenotypic integration of floral and vegetative characters and pollination syndromes].

The general result that there is relatively little plasticity of measures of phenotypic integration is reinforced by this study, considering how most (though not all) total and indirect effects in our model did not vary dramatically in response to the imposed treatments. Some of the paths in our causal model did show plasticity in response to our treatments, occasionally even resulting in a qualitative (change of sign) modification. For example, the total effect of rosette diameter on bolting date (Fig. 5) was positive under low light and standard water, but negative under standard light and flood (it was near zero in the other two environments). However, most changes in the reaction norms of the path diagrams were not this dramatic. Moreover, indirect effects were even less impacted by any change in the environmental conditions.

Of course, at this stage we can only speculate on what underlying genetic and/or hormonal factors may be responsible for these limited shifts in the pattern of phenotypic integration, but such findings should direct future research conducted at a more mechanistic level of analysis, something that is facilitated by *A. thaliana* being a model system for molecular biology and genomics. For example, ongoing quantitative trait loci studies in our laboratory are aimed at identifying some of the genomic regions that contribute to the limited plasticity of integration patterns one observes in *A. thaliana*. Perhaps even more informatively, we will seek the location of genomic regions that underlie the hypothesized causal connections among traits, regardless of the manifest plasticity of such connections.

More stress = more integration?

Contrary to the hypothesis that stress may be a good predictor of the level of phenotypic integration exhibited by an organism, we found no evidence of a connection between the two, despite the fact that some of our treatments were much more stressful (as perceived by

the plants) than others. We have also referred to our own previous work highlighting relatively high stability in the face of environmental variation of patterns of integration in *A. thaliana* (Pigliucci and Kolodynska, 2002a, 2002b). Moreover, Pigliucci and Marlow (2001), also studying *A. thaliana*, found little evidence for changes in the pattern of simple character correlations in response to exposure to cold.

There may be several reasons for the lack of a stress–integration connection in the present study. The first possibility is simply that the accessions of *A. thaliana* that we used display little phenotypic plasticity to light or water availability of individual traits, which translates into lack of plasticity of measures of integration among the same traits. However, although the analyses of variance found very little evidence for genotype × environment interactions, there was ample evidence for plasticity of every trait, as indicated by the large portion of phenotypic variance accounted for by either the light or the water treatments. So what we have here is a situation in which ample plasticity for individual traits is nonetheless coupled with a relatively high degree of across-environment stability of trait interrelationships. Second, it is possible that *all* treatments corresponded to stressful conditions for this species, which would weaken our ability to test the hypothesis of a relationship between stress and integration. However, we did find a large and statistically significant difference in reproductive output (our measure of perceived stress) among our environmental conditions, so at least we can be confident that some of our treatments were *more* stressful than others. Third, it is possible (as proposed by Schlichting and Pigliucci, 1998) that it is not stress *per se* that elicits changes in the degree of integration, but rather how novel an environment is compared with those historically encountered by the species being studied. Even so, one of our stressors (water) was imposed in what ought to be a rather novel way for *A. thaliana*, considering that it rarely, if ever, experiences floods [it does, on the other hand, encounter relatively low light conditions in the field (M. Pigliucci, personal observation)].

The general hypothesis of a connection between environmental stress and patterns of integration has been proposed and discussed by Schlichting (1986, 1989). He reanalysed the data of Marshall *et al.* (1986) for two species of *Sesbania* and found both interspecific differences and responses of phenotypic integration to environmental stress. In animals, work by Badayev and Foresman (2000) has shown that stress-induced patterns of morphological variation in shrew mandibles parallel the observed patterns of evolutionary divergence observed in the same group. Moreover, there also is physiological evidence for the integrated response of morphological characters to stress, both in plants (e.g. Chapin, 1991) and in animals (e.g. Svensson *et al.*, 2002). However, it is unclear whether one can make reasonable generalizations about the stress–integration connection, not the least because of obvious complicating factors such as the different kinds of stress an organism may have evolved (or not) to respond to, the idiosyncrasies of each species' developmental and physiological machinery, and the (often largely unknown) evolutionary ecological history of the taxa being considered. For example, Sinervo and Svensson (2002) have proposed that correlational selection may be responsible for the observation of correlational patterns, although they also point out that the establishment of selection-led linkage disequilibrium at the genetic level is likely to be maintained only if selection is strong and recurrent in time. These and several other complicating factors are exactly what makes the use of a small number of model systems, being studied at different levels and from different angles, a potentially fruitful tool to gain a better understanding of the ecology and evolution of phenotypic integration.

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