Phenotypic convergence along a gradient of predation risk

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A long-standing question in ecology is whether phenotypic plasticity, rather than selection *per se*, is responsible for phenotypic variation among populations. Plasticity can increase or decrease variation, but most previous studies have been limited to single populations, single traits and a small number of environments assessed using univariate reaction norms. Here, examining two genetically distinct populations of *Daphnia pulex* with different predation histories, we quantified predator-induced plasticity among 11 traits along a fine-scale gradient of predation risk by a predator (*Chaoborus*) common to both populations. We test the hypothesis that plasticity can be responsible for convergence in phenotypes among different populations by experimentally characterizing multivariate reaction norms with phenotypic trajectory analysis (PTA). Univariate analyses showed that all genotypes increased age and size at maturity, and invested in defensive spikes (neckteeth), but failed to quantitatively describe whole-organism response. In contrast, PTA quantified and qualified the phenotypic strategy the organism mobilized against the selection pressure. We demonstrate, at the whole-organism level, that the two populations occupy different areas of phenotypic space in the absence of predation but converge in phenotypic space as predation threat increases.

Keywords: phenotypic plasticity; phenotypic trajectory analysis; convergence;
Daphnia pulex; *Chaoborus*; predation

1. INTRODUCTION

A long-standing question in ecology is whether phenotypic plasticity, rather than selection *per se*, is responsible for phenotypic variation among populations. Phenotypic plasticity can, conceivably, either increase large-scale variation in phenotype along selection gradients or generate convergence in phenotype among genetically distinct populations exposed to a common selective pressure. Such patterns result from the ways in which reaction norms, which describe plasticity, manifest across environments. Theoretically, the expression of phenotypic plasticity can generate patterns of divergence or convergence in traits, and at multiple scales.

One of the most pervasive, well-studied selection pressures that might generate such patterns in phenotypic variation is predation risk. Prey species respond to predation risk by altering, often in an adaptive manner, aspects of their phenotype, including life history, morphology and behaviour [1,2]. Because the source (e.g. predator) and magnitude of predation risk can change quickly and unpredictably at multiple temporal and spatial scales, prey species must be able to respond rapidly to changes in environment. Phenotypic plasticity allows rapid and localized response to unpredictable risk.

The importance of predation risk and predator-induced plasticity in generating variation along spatial

gradients of predation risk has been demonstrated. Trussell & Smith [3] showed that large-scale spatial variation in morphology could be better attributed to large-scale gradients of predation risk and inducible defences, rather than selection *per se*. In contrast to this evidence for divergence, there is limited (if any) evidence that phenotypic plasticity can generate convergence in phenotype among genetically distinct populations exposed to a common selective pressure.

Analyses of phenotypic plasticity and its role in trait variation revolve around assessing reaction norms—the change in mean trait value in a genotype as a function of an environmental gradient. Previous research addressing aspects of phenotypic convergence or divergence under predation risk using reaction norm approaches can be broadly separated into two groups. The first group of analyses tends to focus on single traits in a few environments (for a review see [4]) or at most two traits (e.g. [5,6]). These studies demonstrate the potential role of plasticity in generating or reducing variation in a trait among different environments. However, they offer little insight into a more comprehensive understanding of the phenotype gained by explicitly examining covariation among traits.

The second group of studies examines multiple traits (multivariate), multiple environments or both, but are also limited in scope. They tend to focus on a single class of trait (e.g. morphology [7], or life history [6]; but see [8]) and the source but not the magnitude of predation risk. They thus begin to illuminate how combinations of traits function together in a plastic

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phenotype (a complex; cf. [3,9]) and how such a complex phenotype might change when exposed to different selection pressures [8]. But they fail to elucidate how gradients of predation risk may influence, via plasticity, the entire phenotype.

Both types of studies have been instrumental in highlighting predator-induced plasticity as a potential adaptation to environmental variability and a source of phenotypic variation. Furthermore, there is evidence, as noted above, that large-scale spatial variation in traits can be attributed to plasticity (e.g. [3]). However, no study has explicitly examined whether plasticity can be responsible for convergence in phenotypes among different populations.

Here, we test this hypothesis by examining a multivariate trait response (11 traits) in two distinct populations of the water flea *Daphnia pulex* facing fine-scale variation in predation risk from an invertebrate predator, the phantom midge larvae (*Chaoborus flavicans*). Evaluating a multivariate description of plasticity along a fine-scale gradient in individuals from multiple populations allows one to develop a comprehensive, context-dependent description of the response to risk (via well-characterized reaction norms) and begins to characterize the response as a strategy rather than a single trait (e.g. [10,11]).

For example, consider two populations that have experienced different predation regimes. One population has experienced a single predator; the other, multiple predators, including the single predator in the first population (i.e. a shared predator). We might expect the population experiencing multiple predators to possess different phenotypes, and different phenotypic and genetic covariation among traits, compared with the single predator population (e.g. [12,13]). If it does, then we might expect that the response to the shared predator in each population may be different. Alternatively, as we aim to test, evolution may favour a common response to the shared predator, and thus a common phenotypic target (i.e. convergence).

Recently, Adams & Collyer [14] introduced phenotypic trajectory analysis (PTA), an analysis tool designed to assess numerous traits and their covariation at once, and to allow visualization and statistical description of how a complex phenotype changes as the environment does. The technique calculates the trajectory (length, shape and direction) of phenotypic change along an environmental gradient while respecting high-dimensional data (i.e. numerous traits, independent of the graphical representation). The trajectory and associated statistics are the same regardless of the plotted axes. This method generates a reaction norm (the trajectory) for a set of potentially correlated traits.

Differences in trajectory lengths among genotypes establish genetic differences in total phenotypic change (i.e. the slope of a univariate reaction norm); shape changes reflect the recruitment of, or change in, different traits among genotypes along the environmental gradient; and the angle between trajectories indicates variation in starting or ending phenotypes and the associated patterns of convergence (or divergence). It is thus an ideal tool with which to assay divergence or convergence, providing metrics with which to characterize population-specific, multivariate reaction norms.

Here, we use PTA to test for phenotypic convergence through plasticity between two populations. As described above, one population experiences only a single midge predator (*C. flavicans*) and the second population multiple predators (midge and fish; *C. flavicans* and *Gasterosteus aculeatus*). We examine predator-induced plasticity in a set of 11 morphological and life-history traits along fine-scale, realistic gradients of predation risk from the shared predator.

Specifically, we ask three questions. (i) Does each population occupy equivalent or different phenotypes in the absence of predation (i.e. is there natural phenotypic differentiation between populations under benign conditions)? (ii) Relative to phenotypes in the absence of predation, do both populations respond similarly to common predation risk (e.g. does plasticity generate complex, phenotypic convergence)? (iii) Is there genetic variation in complex (multivariate) phenotypic plasticity in each population? Importantly, we ask these questions of the covarying phenotype complex. Our study, using a new method for visualizing and analysing multivariate changes in the traits, is thus one of the first to link multivariate phenotypic plasticity to convergence and history.

2. METHODS

(a) Study system

Daphnia pulex is a freshwater microcrustacean that faces seasonal predation pressure from 'young of the year' fish in spring, and *Chaoborus* midge larvae during the middle and latter parts of the summer. Fish and *Chaoborus* predation risk is manifest as selection (e.g. mortality) and via chemical cues emitted by both predators. Both the foraging predators and their chemical cues produce opposite effects in the life history of *D. pulex*. Fish are large selective predators, selecting larger prey items, which favours daphnids that mature earlier and at a smaller size [15]. Conversely, *Chaoborus* are gape-limited, small selective predators, preying on juvenile daphnids, which favours fast growth in juvenile daphnids (to reach a size refuge; e.g. [16]). Because *Chaoborus* predation is centred on juveniles, there is no predator selection pressure on maturation and *Chaoborus*-exposed individuals mature at a larger size and at a later age [17].

In addition to the life-history responses, *Chaoborus* larvae also generate dose-dependent changes in morphology. The most striking adaptation is 'neckteeth', comprising a neck pedestal and a series of spikes that arise during juvenile (second and third) instars as a result of maternal and embryonic exposure to the kairomone (e.g. [16,18]). The neckteeth, similar to many defences induced in rotifer predation systems (for review, see [19]), confers a survival benefit to juveniles, which are the preferred prey of *Chaoborus* [20,21].

(b) Genotypes and populations

We examined 12 iso-female lineages of *D. pulex* originally collected from two ponds (separated by approx. 8 km) in Sheffield, UK, but maintained in the laboratory for several years. Historically, daphnids collected from pond A (seven genotypes; UK grid reference 436155:389947) had been subjected only to predation from invertebrates (primarily *Chaoborus*), while daphnids collected from pond B (five genotypes; UK grid reference 436200:389900) were also

subject to seasonal predation by fish. Genetic differences between genotypes, and between populations, were confirmed by microsatellite analysis ($F_{st} = 0.39$, using 18 microsatellite loci; J. Stapley, J. Reger, M. J. Carter & A. P. Beckerman 2009, unpublished data).

Daphnids were maintained in hard artificial pond water [22] and fed the algae *Chlorella vulgaris*. Experiments were conducted at 21°C in controlled-temperature rooms on a 16:8 light cycle. For experiments involving predator cue, we extracted kairomone from frozen *C. flavicans* (Honka, Germany), following the method developed by Tollrian [23] (see also [21]).

(c) Traits and environmental gradient

We measured the reaction norms of all 12 genotypes for 11 traits: size at birth, size at risk (second–third instar), size at maturity, age at maturity, number of juvenile instars, lifetime growth rate, growth rate at risk, neckteeth induction, clutch size, tail length and tail angle. To produce a realistic fine-scale gradient of predation risk, we exposed 15 individuals of each genotype independently to seven concentrations of extracted predator cue (0, 0.1, 0.25, 0.5, 0.75, 1 and $2 \mu\text{l ml}^{-1}$).

For each treatment, three third-generation mothers of at least their third brood were exposed to the relevant cue concentration. Five neonates of the subsequent brood from each mother (a total of 15 neonates per genotype) were distributed individually into glass jars containing 50 ml of hard artificial pond water [22], food (2×10^5 cells ml^{-1} algae) and the appropriate concentration of chemical cue. Daily, each animal was photographed, neckteeth induction scored (*sensu* [21,23]) and animals transferred to a new jar containing fresh media and cue. The experiment continued until all animals had reached maturity (the appearance of eggs in the brood pouch).

Body size (the linear distance from the top of the head capsule through the eye to the base of the tail), total size (from the top of the head capsule to the tip of the tail spine), tail length and tail angle were calculated from daily photographs using IMAGEJ [24]. Using these daily data, instar-specific and lifetime growth rates were calculated ($\ln[\text{final} - \text{initial size}]/t_1 - t_0$). As a proxy for fecundity, we counted the number of eggs present in the brood pouch at maturation.

(d) Data analysis

Univariate reaction norms: Reaction norms of neckteeth induction across the predator treatments were analysed with a three-parameter logistic model, using a nonlinear mixed-effects model (see [21,25]), with predator cue concentration as the fixed effect and genotype as a random effect. The three-parameter logistic model is a sigmoid curve with an asymptote (maximum induction), an inflection point (threshold concentration) and a scale parameter (steepness of the inflection). The mixed-effects model approach allows us to determine population average as well as genotype-specific effects of the predator cue on the attributes of the resulting curve. The model was run separately for each population to allow derivation of independent asymptotes, inflection points and scale parameters for each population and for each genotype within populations.

To quantify changes in life-history traits (e.g. size and age at maturity) associated with the predation gradient, we constructed probabilistic reaction norms (PRNs [6,17]). We

evaluated the reaction norms using logistic regression models of the probability of maturation as a function of size and age. PRNs generated by experimentally varying predation risk along a defined fine-scale environmental gradient remove systematic bias that can occur when simply measuring size and age at maturity, and can reveal an accurate representation of the reaction norm [6].

These probabilistic models, specified as a binomial/logistic regression, estimate two of the same parameters (slope and threshold/midpoint) that the three-parameter logistic model does for induction. However, a three-parameter model is over-parametrized for traits where the asymptote is fixed. We modelled maturation as a binary response variable, size or age and the two-way interaction between the predation gradient and population as explanatory variables, and genotype nested in population as random effects. All other traits were analysed with generalized linear mixed models specifying a population by treatment interaction, with genotype nested in population as random effects. This allowed us to generate highly resolved reaction norms for all traits.

(e) Phenotypic trajectory analysis

PTA allows statistical inference for the direction, magnitude and shape of phenotypic change along an environmental gradient and can be conducted on either original data or using scores obtained through ordination methods such as principal components analysis (PCA); both methods produce identical values and statistical inferences, provided that scaling and centring of the data are consistent between approaches. The benefit of using PC scores lies in the ease of visual interpretation of the phenotypic landscape. For this reason, we used PC scores in our analyses and plotted the centroid averages of the PC scores for each genotype at each predator cue level. Thus, prior to the PTA, we performed a PCA on mean centred and scaled trait data (correlation matrix).

The first step in PTA is a multivariate analysis of variance (MANOVA) with the PC scores (or original data; see above) as the response variable and predator environment, genotype and their interaction as the explanatory variables [14].

PTA determines the probability that the observed MANOVA test statistics are greater than would be expected from random pairs of trajectories sampled from a reduced model, where main effects are retained but interactions are removed. It generates probabilities about the direction, shape and magnitude of change by repeatedly randomizing residuals from the reduced model and re-estimating parameters to produce empirical null distributions of trajectory contrasts and correlations.

Are populations different? Are they convergent under increasing predation threat? We measure initial differences and convergence/divergence by explicitly comparing the distance (vector length at each centroid) between populations in each environment along the gradient. This is an extension of the two-state (i.e. vector) approach [26], which did not consider vector lengths between populations in the same environment.

How much variation characterizes the complex phenotype? We applied PTA within each population to measure genotypic variability in phenotypic change. Because there were many more trajectories involved in this analysis, we restricted analysis to pairwise combinations of individual genotypes. We analysed the effects of population, concentration and

Figure 1. Population-specific reaction norms for neckteeth induction. x -axes indicate increasing concentration of predator cue. y -axes indicate extent of morphological induction. In each panel, the solid line is the reaction norm for the population predicted by the model and the dashed line is the mean reaction norm for the genotype.

their interaction using a response variable that was the difference between vector length at each cue concentration and the vector length in the absence of predator cue. For this analysis, three 'populations' were evaluated. The first two arise from pairwise combinations of genotypes within a source population, and the third from pairs that spanned both populations, designated as 'between-population' comparisons.

All analyses were conducted using R v. 2.10.1 [27].

3. RESULTS

(a) *Univariate analyses*

(i) *Induced defence (neckteeth)*

We detected substantial variation in morphological defences within and between populations (figure 1). Genotypes from pond A were more variable, sensitive (earlier inflection point) and reactive (larger asymptote), but less plastic (steeper slope), than genotypes from pond B (figure 1 and table 1).

(ii) *Life history and general morphology*

As predicted by theory and seen in many systems, genotypes from both populations increased both age and size at maturity following exposure to *Chaoborus* kairomones (figure 2) [28]. Analysing both size and age, the effect of cue concentration on the probability of maturation depended on population (size model: $t_{\text{treatment} \times \text{pond}} = 14.9$; age model: $t_{\text{treatment} \times \text{pond}} = 5.1$; table 1 and figure 2).

Table 1 summarizes the effects of treatment and pond on the remaining eight traits. The effect of treatment on growth rates and clutch size depended on pond. Treatment and pond were additive for a number of instars. Tail length varied only with treatment, while tail angle, size at birth and size at risk (second–third instar) varied only with pond.

(b) *Multivariate analyses*

(i) *Principal components analysis*

Positive associations were detected between (i) age at maturity and moult number, (ii) size at maturity and clutch size, (iii) juvenile size measurements, and (iv) overall growth rate and during the risk period.

The first principal component (variance 29.9%) was negatively associated with age and number of instars to maturity, and positively with growth rate and size during the predation risk window. The second principal component (20.8%) was positively associated with size at maturity, size at birth, clutch size and neckteeth. We characterize PC1 as a generalized growth axis and PC2 as a size axis.

MANOVA applied to the PC scores showed that the effect of cue concentration on the multivariate response depended on population or genotype (table 2).

(ii) *Phenotypic trajectory analysis*

Are populations different? Each population had a unique phenotypic trajectory (figure 3a and table 3). Both populations (figure 3b,c) exhibited similar amounts of phenotypic change ($\Delta \text{length} = 0.564$, $p = 0.381$), but differed in direction ($\theta = 36.4$, $p < 0.001$) and shape (shape = 0.684, $p < 0.001$).

Are populations convergent under increasing predation threat? The distance between centroids decreased with increasing predator cue up to $0.5 \mu\text{l ml}^{-1}$ but then stabilized, indicating that populations became more similar as predator cue increased up to a threshold (figures 3a and 4a; electronic supplementary material, table S1).

(iii) *Genetic and population variation in the complex phenotype*

Within populations, all genotypes had unique trajectories. In pond A (figure 3b)—the single-predator, fishless

Table 1. Mean trait values ($\pm$ s.e.) and *F*-statistics for each measured trait in populations from *Chaoborus*-only (pond A) or *Chaoborus*-and-fish (pond B) populations. Means were estimated from linear models (trait $\sim$ cue concentration $\times$ population). Degrees of freedom were 3 and 1204 for all models. Asterisk (*) denotes significance of the explanatory variables.

	size (mm)				
	maturity ¶	birth	risk period	age ^a	clutch size
treatment	68.1***	3.3	0.8	45.1***	25.4***
population	19.8***	25.7***	155.2***	4.2*	0.9
interaction	14.9***	0.4	3.6	5.1*	7.5**
pond A (no fish)					
no predator cue	1.80 (0.07)	0.69 (0)	1.04 (0)	6.18 (0.86)	8.85 (0.13)
0.1 $\mu\text{l ml}^{-1}$	1.81 (0.07)	0.69 (0)	1.04 (0)	6.24 (0.85)	8.88 (0.03)
0.25 $\mu\text{l ml}^{-1}$	1.81 (0.06)	0.7 (0)	1.03 (0)	6.33 (0.82)	8.93 (0.08)
0.5 $\mu\text{l ml}^{-1}$	1.82 (0.06)	0.7 (0)	1.03 (0)	6.47 (0.80)	9.00 (0.15)
0.75 $\mu\text{l ml}^{-1}$	1.83 (0.06)	0.7 (0)	1.03 (0.01)	6.61 (0.77)	9.08 (0.23)
1 $\mu\text{l ml}^{-1}$	1.83 (0.06)	0.7 (0)	1.03 (0.01)	6.73 (0.74)	9.15 (0.3)
2 $\mu\text{l ml}^{-1}$	1.85 (0.05)	0.7 (0.01)	1.02 (0.02)	7.14 (0.65)	9.46 (0.61)
pond B (fish and invertebrates)					
no predator cue	1.74 (0.06)	0.68 (0.02)	0.97 (0.06)	6.58 (0.86)	8.59 (0.26)
0.1 $\mu\text{l ml}^{-1}$	1.75 (0.06)	0.68 (0.02)	0.97 (0.06)	6.61 (0.85)	8.68 (0.17)
0.25 $\mu\text{l ml}^{-1}$	1.76 (0.06)	0.68 (0.02)	0.97 (0.06)	6.65 (0.83)	8.82 (0.03)
0.5 $\mu\text{l ml}^{-1}$	1.78 (0.05)	0.68 (0.01)	0.98 (0.06)	6.72 (0.80)	9.05 (0.2)
0.75 $\mu\text{l ml}^{-1}$	1.80 (0.05)	0.68 (0.01)	0.98 (0.06)	6.78 (0.77)	9.27 (0.42)
1 $\mu\text{l ml}^{-1}$	1.82 (0.05)	0.68 (0.01)	0.98 (0.06)	6.83 (0.74)	9.50 (0.65)
2 $\mu\text{l ml}^{-1}$	1.87 (0.04)	0.69 (0.01)	0.98 (0.06)	7.03 (0.65)	10.42 (1.57)
	growth rate				
	lifetime	risk period	no. instars	tail size (mm)	tail angle
treatment	58.6***	31.9***	95.2***	27.5***	2.8
population	17.1***	3.6	31.2***	0.02	377.6***
interaction	21.1***	4.2*	1.6	2.5	1.9
pond A (no fish)					
no predator cue	0.18 (0)	0.23 (0)	5.36 (0.03)	0.24 (0)	182 (0.22)
0.1 $\mu\text{l ml}^{-1}$	0.18 (0)	0.22 (0)	5.38 (0.02)	0.24 (0)	182 (0)
0.25 $\mu\text{l ml}^{-1}$	0.18 (0)	0.22 (0)	5.42 (0.06)	0.24 (0)	182 (0)
0.5 $\mu\text{l ml}^{-1}$	0.18 (0.01)	0.22 (0.01)	5.48 (0.12)	0.25 (0)	182 (0)
0.75 $\mu\text{l ml}^{-1}$	0.17 (0.01)	0.21 (0.01)	5.54 (0.17)	0.25 (0)	182 (0.01)
1 $\mu\text{l ml}^{-1}$	0.17 (0.01)	0.21 (0.02)	5.59 (0.23)	0.25 (0.01)	182 (0.01)
2 $\mu\text{l ml}^{-1}$	0.16 (0.03)	0.19 (0.04)	5.83 (0.46)	0.25 (0.01)	182 (0.02)
pond B (fish and invertebrates)					
no predator cue	0.17 (0.01)	0.23 (0)	5.55 (0.19)	0.24 (0)	187 (4.82)
0.1 $\mu\text{l ml}^{-1}$	0.17 (0.01)	0.22 (0)	5.57 (0.21)	0.24 (0)	187 (4.77)
0.25 $\mu\text{l ml}^{-1}$	0.17 (0.01)	0.22 (0)	5.59 (0.23)	0.24 (0)	187 (4.69)
0.5 $\mu\text{l ml}^{-1}$	0.17 (0.01)	0.22 (0)	5.64 (0.28)	0.24 (0)	187 (4.57)
0.75 $\mu\text{l ml}^{-1}$	0.17 (0.01)	0.22 (0.01)	5.68 (0.32)	0.25 (0)	186 (4.44)
1 $\mu\text{l ml}^{-1}$	0.17 (0.01)	0.22 (0.01)	5.73 (0.37)	0.25 (0.01)	186 (4.31)
2 $\mu\text{l ml}^{-1}$	0.17 (0.02)	0.21 (0.02)	5.91 (0.54)	0.26 (0.02)	186 (3.81)
neckteeth development	inflection point	slope	asymptote		
pond A (no fish)	0.13 (0.008)	0.05 (0.003)	73.9 (2.36)		
pond B (fish and invertebrates)	0.37 (0.108)	0.08(0.019)	58.2 (3.88)		

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

^aInflection point and slope in parentheses estimates from binomial generalized linear models (see text).

pond—genotypes differed in amount (Δ length 0.156, $p < 0.001$), direction ($\theta_{\text{pond A}} = 125.8$, $p < 0.001$) and shape (shape = 0.007, $p = 0.001$) of phenotypic change (table 3).

By contrast, genotypes from the two-predator pond (pond B; figure 3c) differed only in the direction of phenotypic change ($\theta_{\text{pond B}} = 154.9$, $p < 0.001$), but not in the amount of change (Δ length 0.139, $p = 0.074$) or the shape of the trajectory (shape = 0.003, $p = 0.109$; table 3).

Phenotypes in the single-predator pond (A) became more dissimilar as predator cue increased (Δ between =

5.01, $p < 0.001$; figures 3b and 4b, electronic supplementary material, table S1). By contrast, there was no evidence of either convergence or divergence in the two-predator pond (B) (Δ between = 0.088, $p = 0.94$; figures 3c and 4c; electronic supplementary material, table S1).

4. DISCUSSION

Ecologists and evolutionary biologists have long been interested in whether phenotypic plasticity is a

Figure 2. Population-level probabilistic reaction norms for (a,c) age and (b,d) size at maturity at different predator cue concentrations in each population ((a,b) pond A; (c,d) pond B)). Data are based on model predictions from a generalized linear model. Solid line represents no predator cue. Increasing thickness of dashed lines indicates increasing predator cue concentration.

Table 2. Summary statistics from MANOVA conducted on 11 traits at the population, genotype and population-specific genotype levels.

	Pillai's trace	F	num,den d.f.	p
<i>between populations</i>				
cue concentration	0.835	17.9	66,7134	<0.0001
population	0.405	73.3	11,1184	<0.0001
cue × population	0.167	3.1	66,7134	<0.0001
<i>between genotypes</i>				
cue concentration	0.99	20.1	66,6714	<0.0001
genotype	2.60	31.7	121,12364	<0.0001
cue × genotype	1.90	3.6	726,12364	<0.0001
<i>pond A</i>				
cue concentration	1.082	13.0	66,3888	<0.0001
genotype	2.137	32.6	66,3888	<0.0001
cue × genotype	1.719	3.4	396,7183	<0.0001
<i>pond B</i>				
cue concentration	1.076	9.3	66,2796	<0.0001
genotype	1.655	29.8	44,1856	<0.0001
cue × genotype	1.618	3.4	264,5181	<0.0001

mechanism by which genetically distinct populations might converge or diverge phenotypically under common selection pressure. Ultimately, such patterns are about expressed phenotypic variation. Under convergence (or divergence), the phenotypic variation facing selection pressures is dramatically reduced (increased) and, in the case of divergence, may facilitate ecological speciation. These patterns of plasticity-induced variation

thus reflect past selection pressures and the opportunities for further evolution, and may contribute to our understanding of mechanisms controlling ecological differentiation [11,29,30].

Because plasticity is a whole-organism trait, detecting convergence or divergence is fundamentally a multivariate issue. Here we made use of a new multivariate technique, PTA, for assessing convergence or divergence under predation risk using a model predator–prey system of *D. pulex* facing predation risk by the small-size-selective predator *C. flavicans*. We examined the multivariate phenotypic trajectory of two genetically distinct populations, reflecting different predation regimes to show that they converge along a gradient of common selection pressure from different starting points. We then examined genotype-specific trajectories to examine contributions of each genotype to expressed variation.

An examination of the standard univariate reaction norms for 11 traits demonstrated that a ‘multiple but univariate’ approach is interesting but not necessarily illuminating. The univariate trait analyses revealed substantial genetic variation in phenotypic plasticity for each trait. As expected, all genotypes responded by increasing age and size at maturity, and investing in neck-teeth during early instars. In addition, genotypes made other phenotypic adjustments in response to predator cue, and trait expression varied between populations. This approach tells us that genotypes of *D. pulex*, across populations, respond similarly to predation pressure in some core traits—something we also know from meta-analyses [28]. However, it does not quantify or synthesize the multivariate change in phenotype or the magnitude of

Figure 3. PCA plots showing the relationship between traits (labelled) with overlaid phenotypic trajectories between (a) populations, (b) pond A genotypes and (c) pond B genotypes. Shaded circles indicate predator cue concentration. White, no cue; grey, intermediate concentrations ($0.1\text{--}1\ \mu\text{l ml}^{-1}$); black, the highest tested concentration ($2\ \mu\text{l ml}^{-1}$).

Table 3. Comparisons of phenotypic trajectories between and within populations. θ is the angular difference between trajectories, Δlength is the difference in length of each trajectory and shape reflects differences in shape of the trajectories. $\Delta\text{between}$ indicates the difference in Euclidean distance between groups (centroids; pond/genotypes) at each cue concentration. The $\Delta\text{between}$ value reported is the difference between the centroid averages in the absence of cue ($0\ \mu\text{l ml}^{-1}$) and the maximal tested concentration ($2\ \mu\text{l ml}^{-1}$). Full $\Delta\text{between}$ values for all comparisons can be found in the electronic supplementary material, table S1. p -values represent the probability of finding a larger test statistic under a null distribution.

comparison	θ	p	Δlength	p	shape	p	$\Delta\text{between}$	p
pond A–B	164.5	0.001	1.875	0.005	0.006	0.007	1.104	<0.001
in pond A	125.8	<0.001	0.156	<0.001	0.007	0.001	5.01	<0.001
in pond B	154.9	<0.001	0.139	0.074	0.003	0.109	0.088	0.939

convergence (divergence). Fundamentally, it fails to quantify or qualify the phenotypic strategy—the integration of traits—that the organism mobilizes against the selection pressure.

In contrast to this univariate assessment, PTA quantifies and qualifies the phenotypic strategy—the multivariate reaction norm—that the organism mobilizes against the selection pressure. PTA helps to visualize and statistically characterize the direction, shape and magnitude of phenotypic change among all 11 traits. Here, we demonstrated quantitative, multivariate phenotypic convergence along a gradient of predation threat shared by two distinct populations. Our analysis revealed a threshold-like convergence (figure 4a; electronic supplementary material, table S1). We further show that substantial genetic variation in trajectory underpins the average convergence at the population level, with phenotypic divergence demonstrated among genotypes in the fishless, single-predator population.

(a) Univariate considerations

Univariate analyses highlighted many trait-specific differences between the two ponds. With respect to morphological defences, all genotypes differed in their sensitivity to *Chaoborus* kairomone and in their maximal response. However, genotypes from the single-predator, fishless pond (pond A) produced a greater change in morphology, and at a lower concentration (i.e. they were more sensitive and responsive to *Chaoborus* kairomones), than genotypes from the multiple-predator

population (pond B), which also experiences seasonal predation by fish. This may indicate that genotypes from pond B are constrained in their response to *Chaoborus* by their ability (need) to respond to other predators. Contrasting predators can constrain morphological responses and potentially influence diversification [31].

These univariate analyses suggest a potential influence of historical predation regime (table 1). Genotypes from the fishless pond respond with less variation in terms of morphology and increase age more than size in response to *Chaoborus* kairomone. In contrast, genotypes from the fish and *Chaoborus* pond are more variable in morphology, and increase size more than age. However, trait investment in the fishless pond is biased towards age (approx. 15% increase) over size (3% increase), but in the fish population investment in these traits is roughly equal (approx. 7.5% and 6.7% for size and age, respectively). We suggest that it is constraints imposed by recent selection history that explain the different responses of the same traits between populations. We also suggest that the PTA provides deeper insight into such constraints, as well as alternative explanations.

(b) Phenotypic trajectory analysis

Only by considering the phenotype in a multivariate context along a gradient of predation risk, and not a series of univariate traits in simple presence/absence conditions, can we begin to paint a cohesive picture of how plasticity mediates response to common predation risk and the

Figure 4. Pairwise comparisons of distance (mean $\pm$ s.e.) between genotype trajectories between and within populations. (a) The left y-axis represents the length of the vector (arb. units) between centroids at each cue concentration, and the right y-axis represents the difference in vector length between centroids at each cue concentration compared with the control vector length. The solid line represents a Loess curve through the data and shows that convergence peaks at $0.5 \mu\text{l ml}^{-1}$. (b–c) The y-axis represents the difference in vector length between centroids at each cue concentration compared with the control vector length. Negative values indicate phenotypic convergence, while positive values indicate phenotypic divergence. (a–c) Black points and error bars are means $\pm$ s.e. from the original data. Grey regions are fitted values and 95% CI from the model.

expression of genetic variation. PTA helped to visualize multivariate phenotypic plasticity in a statistically robust manner. The ponds clearly converge in phenotype (the trajectories are not parallel), indicating that under a common selective force (i.e. *Chaoborus* predation) similar phenotypes were generated. By overlaying the trajectories onto the PCA phenotypic landscape, rather than just the data cloud (cf. [14]), some understanding of the nature of the phenotypic change is immediately clearer. As predation threat increases, the population trajectories converge towards a common strategy of greater age and size at maturity and increased investment in neckteeth, all at the expense of growth rates. The increase in age and size at maturity, are together a reflection of the diversion of resources towards somatic growth at the expense of development, which results in more time taken to complete the development. That is, growth rate becomes decoupled from development rate (see [17]). The increase in number of instars is a consequence of this decoupling. The concurrent investment in neckteeth during juvenile growth also represents further resource allocation towards somatic growth rather than development.

Our analysis also reveals the nature of the observed convergence. As predation risk increased, convergence occurred rapidly up to a threshold ($0.5 \mu\text{l ml}^{-1}$; figure 4a; electronic supplementary material, table S1). Interestingly, convergence peaks at higher concentrations than the threshold for neckteeth production (inflection point in table 1 and figure 1), the principle morphological defensive strategy employed by *D. pulex* in response to *Chaoborus* predation, but it does peak. In contrast, other traits that contribute to the threshold-like convergence of the whole phenotype (e.g. size and age) do not respond

in a threshold-like manner: they continue to change linearly with increasing predator cue. This indicates that the phenotypic response of the whole phenotype involves multiple traits that converge in different fashions. Moreover, each genotype employs a different trait combination, both qualitatively and quantitatively, in response to increasing predation threat (the shape of each trajectory is different). One could imagine alternative patterns of convergence (e.g. linear or delayed), dependent on the types of univariate trait responses considered. Such an interpretation of multivariate phenotypic change, in the context of major theories about threshold traits, is not possible with univariate methods.

Our analysis elucidates the simultaneous trait changes among individual genotypes, accounts for trait covariation and shows that predator cues direct phenotypic expression towards a point in the phenotypic landscape irrespective of the position of the phenotype in the absence of the cue. While changes in several traits have previously been observed (e.g. [32]), or could be extracted from comparisons of many univariate analyses, it was not previously possible to determine whether the phenotypic changes were in the same direction but without regard to the starting point of the trajectory, or whether the phenotypic changes were directing entire phenotypes towards a point in phenotypic space without regard to the starting position. This is the value of PTA.

(c) On variation in convergence

The convergence observed between populations contrasts with phenotypic divergence within one of our populations

(the *Chaoborus*-only, fishless population; pond A), but not the other (the pond that contains both *Chaoborus* and fish; pond B). Effectively, by performing analysis on genotypes within each population, we assessed the evidence for multivariate genetic variance. We suggested above that variation has not only been maintained within the single-predator population (pond A), but is also divergent with increasing predator cue (i.e. selection strength). Such an argument centres around the idea that pond A experiences only single-predator constraints (directional selection) rather than multiple-predator constraints (i.e. stabilizing selection). In pond B, where previous exposure to multiple types of predation (e.g. fish and invertebrates) necessitates that genotypes are able to respond to either predator (or both), there appears to be a constraint on phenotypes in the absence of any predator cue. In the absence of predator cues, genotypes from this pond mature earlier and smaller than in pond A, which suggests that natural selection has operated to produce intermediate genotypes that through plasticity can respond rapidly to either predator.

Thus, by using PTA in combination with our natural extension to compare among genotypes at each predator concentration, we see evidence of selection acting differently at different scales, producing convergence among, and divergence within, populations as predator threat increased. It appears, then, that selection history in the source populations may dictate the type of response observed. Single-predator history produced divergent phenotypes, while multiple-predator history prevented such divergence, possibly because of constraints in the ability of genotypes to respond to predators that favour opposing strategies. Despite the differences, at the population level, morphological defences (neckteeth) and age and size at maturity all increase at the expense of growth. Only using multivariate techniques (such as PTA), combined with fine-scale exposure gradients, are we able to capture and adequately resolve the variation in complex phenotypic responses at different scales.

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