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Evolution of a predator-induced, nonlinear reaction norm

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Inducible, anti-predator traits are a classic example of phenotypic plasticity. Their evolutionary dynamics depend on their genetic basis, the historical pattern of predation risk that populations have experienced and current selection gradients. When populations experience predators with contrasting hunting strategies and size preferences, theory suggests contrasting micro-evolutionary responses to selection. *Daphnia pulex* is an ideal species to explore the micro-evolutionary response of anti-predator traits because they face heterogeneous predation regimes, sometimes experiencing only invertebrate midge predators and other times experiencing vertebrate fish and invertebrate midge predators. We explored plausible patterns of adaptive evolution of a predator-induced morphological reaction norm. We combined estimates of selection gradients that characterize the various habitats that *D. pulex* experiences with detail on the quantitative genetic architecture of inducible morphological defences. Our data reveal a fine scale description of daphnid defensive reaction norms, and a strong covariance between the sensitivity to cues and the maximum response to cues. By analysing the response of the reaction norm to plausible, predator-specific selection gradients, we show how in the context of this covariance, micro-evolution may be more uniform than predicted from size-selective predation theory. Our results show how covariance between the sensitivity to cues and the maximum response to cues for morphological defence can shape the evolutionary trajectory of predator-induced defences in *D. pulex*.

1. Introduction

Predator-induced defences remain one of the core examples of phenotypic plasticity in the wild. Examples include dramatic changes in morphology, large- and small-scale shifts in habitat use and foraging, and change to the magnitude and direction of several life-history traits [1–8]. Several decades of research in aquatic communities show that plasticity in all these types of traits can be mobilized by exposure to predation risk cues—typically chemicals released by predators [8–17].

One of the most studied examples of adaptive phenotypic plasticity is the predator-induced morphological defence in waterfleas facing predation risk by fish or midge larvae [2,18,19]. *Daphnia pulex* is a species that faces predation risk from at least two size-selective predators: midge larvae and fish. Midge predation is typically small size-selective and results in morphological defences—called neckteeth—at the 2nd/3rd juvenile instar, the size at which they are most at risk. This is accompanied by delayed maturation at a larger size representing investment into growth over reproduction. By contrast, fish predation is typically large size-selective and results in no morphological defence in *D. pulex*, but accelerated maturation at a smaller size, indicative of investment into reproduction over growth [20]. The induced morphological defence in *D. pulex* is considered adaptive, conferring a 30–50% increase in survival [9,21].

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Historically, this predator-induced morphological defence has been classified as a threshold or binary trait [22,23]. However, recent theory [24] and empirical data [8,15,19,25,26] suggest that the morphological defence can be effectively characterized by a continuous, nonlinear function, typically sigmoid, of predation risk [8,25]. In fact, given this functional form, it is possible to characterize population patterns of, and genetic variation in, the induced defence in terms of the parameters of a three-parameter sigmoid model [8,25,27]: the asymptote represents the maximum amount of defence, the inflection point the 'threshold' or sensitivity to predation cues, and the slope or scale parameter the 'reactivity', or how binary the response is. The reaction norm—how the defence varies with increasing predation risk—can be captured by three variables.

Such a characterization allows for a unique game, where we can ask how inducible defences, and the reaction norm that describes them, might evolve. Theory and data suggest that the evolution of a defence characterized by, for example, three traits, will be driven by a combination of how selection pressures target the traits, the heritability of the traits (genetic variation) and correlations among traits (trade-offs and constraints) [6–8]. Here we predict plausible patterns of micro-evolution of the *D. pulex* predator-induced morphological defence, specifically revealing how genetic (co)variation among the amount of defence, the sensitivity to the cue and the reactivity to the cue might constrain such a response. We do this by integrating field-based estimates of genetic variation in the three traits (asymptote, threshold, reactivity) with various, plausible selection regimes via the multivariate breeder's equation. Via these data and the breeders equation, we evaluate the potential micro-evolution of morphological defence reaction norms.

Our approach to make quantitative predictions about the magnitude and direction of the possible responses to selection involves four steps. First, we characterize the reaction norms for morphological predator defence as a sigmoid, three-parameter (trait) reaction norm. Second, we estimate the *G*-matrix that defines a plausible empirical sample of the variance and covariance among the three traits in nature. Third, we derive a set of five plausible selection gradients that combine selection gradient analysis of reproduction via traditional multiple regression tools [28,29] with additional information on survival drawn from size-selective predation theory. Together these data form a data platform on which we can explore potential micro-evolutionary change in reaction norms. To this end, we then combine the *G*-matrix and selection gradients via the multivariate breeder's equation to make predictions about, and visualize, plausible responses to selection by the nonlinear reaction norm of inducible morphological defence.

2. Material and methods

(a) Study system

We characterized the neckteeth reaction norms of and genetic covariance matrix formed by 12 iso-female lineages of *D. pulex* [25], a keystone herbivore of algae in ponds and lakes [30]. In the UK, *D. pulex* can be subject to contrasting and seasonal predation pressure by fish in spring, and midge larvae during summer and autumn [8]. The iso-female clones were originally collected from two very different shallow source ponds in Sheffield, UK, separated by approximately 8 km [25]. Bagshaw pond (seven genotypes; 53°20′5.37″ N, 1°27′8.12″ W) contains only invertebrate predators,

primarily *Chaoborus* sp. (onwards: midge pond). Crabtree pond (five genotypes; 53°24′17.46″ N, 1°27′26.81″ W) contains both fish and midge predators (onwards: fish–midge pond). Genetic differences between iso-female lines were confirmed by microsatellite analysis [31].

(b) Reaction norms

We experimentally determined the predator-induced morphological reaction norm in controlled temperature rooms at 21°C on a 16 L : 8 D cycle. We estimated the reaction norms of the neckteeth by exposing replicate daphnids of each genotype to a gradient of chemical kairomone cues from *Chaoborus flavicans* [8,25]. We extracted kairomone from frozen *C. flavicans*, (Honka, Germany) [8,9,25]. Third-generation mothers of each genotype, at their second brood, were exposed to each concentration of chemical cue (to avoid maternal and grand-maternal effects). Five neonates (third brood, third generation) from each of three mothers, for a total of 15 neonates per genotype, were distributed individually into glass jars containing 50 ml of hard artificial pond water [32], food (*Chlorella vulgaris*; 2×10^5 cells ml^{-1}) and the appropriate volume of purified cue.

We defined the reaction norm along a gradient of seven concentrations of extracted predator cue (0, 0.1, 0.25, 0.5, 0.75, 1, 2 μl ml^{-1}), with the exception of clone 'carlos' that was evaluated at four more concentrations (0.15, 0.4, 0.6, 0.8 μl ml^{-1}). Media were replaced daily and induction of neckteeth was scored following established methods. Each individual was examined under the microscope daily for evidence of induction, from birth to maturation. Points were assigned to the presence of a pedestal and/or the presence of spikes. Pedestals were classified as absent (score = 0), small (score = 30) and large (score = 50). Spikes were assigned 10 points each. For individuals exhibiting neckteeth in multiple instars, we used the maximal amount of induction in analyses, regardless of the instar in which it was observed. Typically, this was either 2nd or 3rd instar. Our maximum level of induction among all replicates was 130 points (large pedestal, eight spikes). All individual scores were normalized to this maximum defining induction levels between 0 and 100 [8,19,25].

We characterized the reaction norms of neckteeth induction for each maternal replicate of each genotype by a sigmoid curve where the asymptote corresponds to maximum induction (maximum), the inflection to the concentration of cue where 50% induction occurs (threshold or sensitivity) and the slope (reactivity), corresponding to the rate of induction or how binary the reaction norm looks [8,25]. These data were estimated for each maternal replicate (i.e. three mothers; $n = 5$ reps/mother × 7 treatments) via nonlinear least-squares with a three-parameter logistic model. Parameters were estimated using the nls function and the associated three-parameter logistic model in R [33]. This analysis provided discrete and replicated trait data for each genotype (3 maternal lines × 12 clones = 36 estimates of maximum, sensitivity and reactivity), along the experimental gradient.

(c) Fitness proxy: reproduction

The experimental exposure of all replicates to predation cues continued until all animals had reached maturity. Maturation was classified as the appearance of eggs in the brood pouch. We defined a proxy of fitness here as a function of age at first reproduction and clutch size and used this in the selection gradient analysis below:

$$R_0 = \frac{\ln(\text{first clutch size})}{\text{age at first reprodcution}}. \tag{2.1}$$

This measure of fitness is focused on reproduction and is highly correlated with population growth rate in exponentially growing populations [34]. Furthermore, our own data confirm

Table 1. Hypothetical output of response of selection in *D. pulex* two predator environments with different scenarios of survival selection gradients on neckteeth reaction norm parameters.

	parameters of neckteeth induction		
	maximum	**threshold**	**steepness**
adaptive strategies	high β: 1	low β: -1	high β: 1
midge pond	higher expression of morphological response, survival benefit	lower sensitivity morphological response, less response costs	faster change
adaptive strategies	low β: -1	high β: 1	low β: 0
fish−midge pond	lower expression of morphological response, less energetic cost	higher sensitivity morphological response, higher response cost	slower change

that first clutch reproduction is highly correlated with reproduction after three clutches ($r = 0.77$, $p < 0.001$; MI Lind, K Yarlett, AP Beckerman 2014, unpublished data).

(d) *G*-matrix of neckteeth phenotypic plasticity

We used the 36 estimates of each of the three traits among the genotypes, structured by maternal identity, to characterize a *G*-matrix. We fit a trivariate model with mother ($n = 12$) as the random effect, using Bayesian Monte Carlo Markov chain (MCMC) generalized linear mixed models (*MCMCglmm* package in R v. 3.3.1 [35]) to obtain the (broad-sense) genetic covariance matrix. We used informative, parameter expanded priors, 180 000 iterations, a burn-in period 45 000 and thinning interval 200 to estimate a joint posterior distribution of $n = 1000$. The chains were well mixed; time-series plots showed no sign of autocorrelation. This produced a modal estimate of variance and covariance among traits and allows direct estimates of broad-sense heritability and genetic correlations. Significance of genetic parameters was assessed using 95% credible intervals calculated from the joint posterior distribution [35]. We estimated the *G*-matrix as the posterior mode of the joint posterior of the variance covariance matrix.

We fit two additional models. First, we removed the random effect. The deviance information criteria (DIC) confirmed that fitting the random component estimated a substantial amount of variation. Second, we tested the significance of covariance structure by comparing a model setting the off diagonal elements of the *G*-matrix to zero using the 'idh()' function instead of 'us()' function for the random effect (see *MCMCglmm* package in *R* v. 3.3.1 [35]). Again, a DIC comparison indicated significant covariance.

(e) Defining selection gradients

Our approach for estimating selection gradients follows [27]. Selection under predation risk in nature depends upon both reproduction and survival. Their relative importance may depend upon the predation regime and how predation risk varies over a season [35]. We, therefore, defined five plausible composite selection gradients, each representing a different weighting of reproduction (β_R) and survival (β_S): β_R, $\beta_R + 0.5\beta_S$, $\beta_R + \beta_S$, $0.5\beta_R + \beta_S$ and β_S (also see Lind *et al.* [27]).

We defined the selection gradient on reproduction (β_R) by regressing our fitness proxy (equation (2.1)) against the three traits defining the reaction norm [28,36]. We fit the selection gradient (fitness proxy as a function of the maximum (asymptote), sensitivity (inflection) and reactivity (scale)) as a response surface using the *rsm* package in *R* [37], which provided estimates of the linear (β; directional selection) and quadratic (γ; indirect selection) components. As has been suggested in the literature [38,39], we

used a randomization test of significance for all parameters of the selection gradients. We re-estimated selection gradients from new regressions based on random allocations of fitness to predictor variables [40]. From this, we derived two-tailed probabilities of estimating selection gradients of the observed magnitudes by chance. We performed separate randomization tests of linear and nonlinear gradients in each environment

We doubled the nonlinear coefficient of multiple regression (following Stinchcombe *et al.* [41]) to standardize their magnitude with the linear and correlational gradients estimated above, which helps to avoid underestimation of disruptive or stabilizing selection. We then explored the extent of nonlinear selection by performing a canonical correspondence analysis. This method helps to identify the major axes of the overall response surface [42]. We used randomization to test the significance of all parameters of β_R, taking the potential non-independence of residual into account [38].

The selection gradients on survival, β_S were defined from the empirical and theoretical literature of size-selective predation on *Daphnia*. This literature (see [20] for theory) is strongly focused around assumptions that gape-limited predators, such as *Chaoborous* larvae, which are able to attack small prey [43,44], leading to conspicuous defences in *D. pulex* [14,26], whereas visually hunting predators such as fish target large prey [44,45] and do not favour induced morphological defences in *D. pulex*. To reflect this, we define the β_S by how each reaction norm parameter would respond in each predation context. For example, we assume that survival is increased under small size-selective midge predation by increasing the asymptote. Table 1 provides an overview of how we characterized adaptive strategies associated with each predation regime, defined by the three variables.

Finally, we created the composite selection gradients by standardizing β_R and β_S to a total length (strength of selection) of 1 and producing the several combinations of reproduction (β_R) and survival (β_S) defined above. Each composite β was standardized to a length of 1 to enable meaningful comparison of the response to selection in the following step (electronic supplementary material, table S1).

(f) The evolution of a reaction norm of inducible defence

We applied the multivariate breeders equation to the phenotypic reaction norm for each of midge and fish−midge population, demonstrating how the *G*-matrix we estimated, along with five different weightings of selection on reproduction and survival, might drive evolution of each reaction norm.

The total response to selection for each component of the reaction norm can be decomposed into direct and correlated

midge – fish

midge
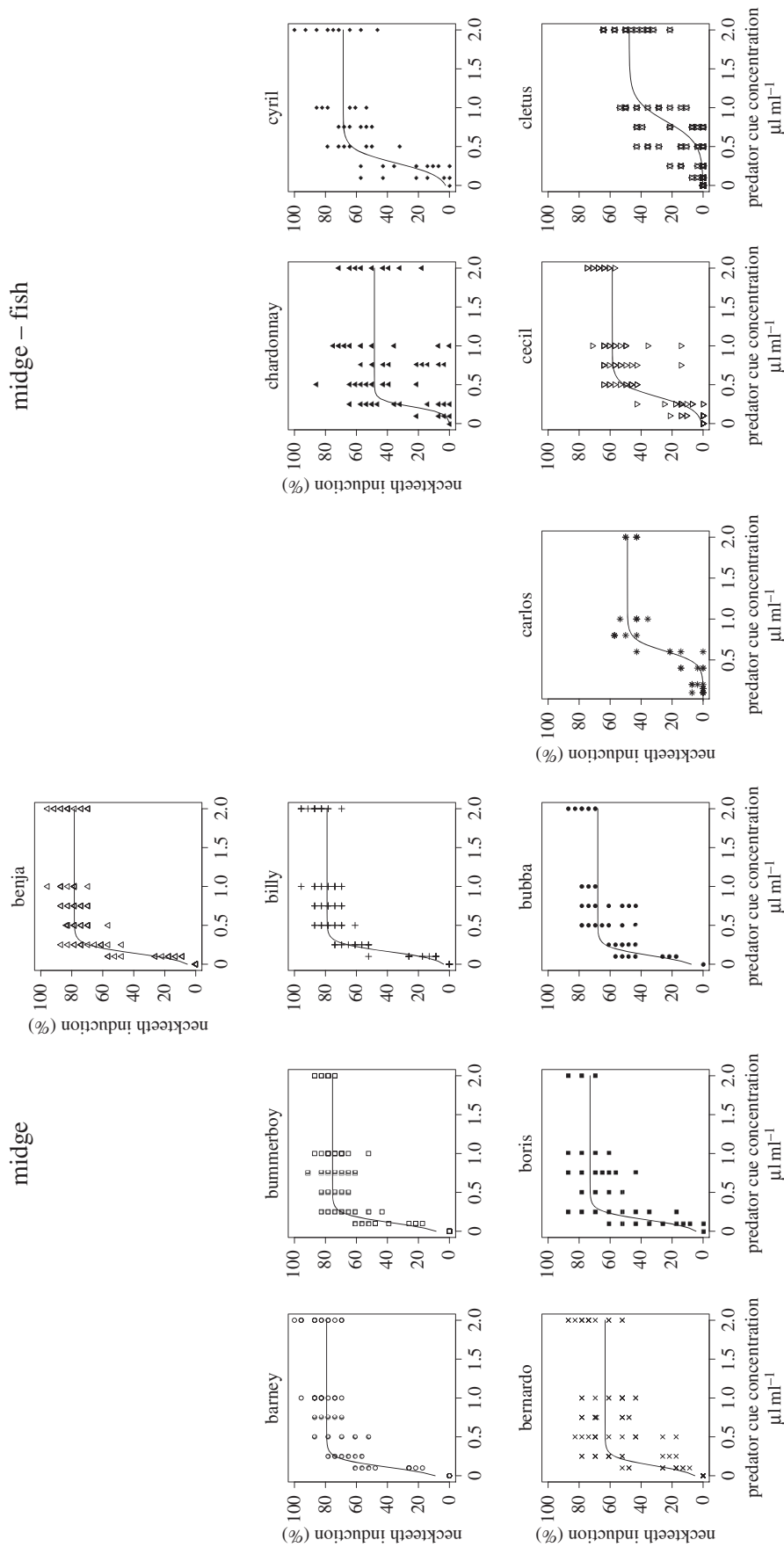
Figure 1. Neckteeth reaction norms of 12 *Daphnia pulex* clones facing a gradient of *Chaoborus* predation risk. Each line corresponds to a clone-specific sigmoid fit that clone's data. The data points correspond to individual replicates of each clone in each experimental condition. We fit a three-parameter sigmoid model where the asymptote is the maximum induction, the inflection is the cue concentration at which 50% induction is reached and the slope or scale is the reactivity or how the binary is the response.
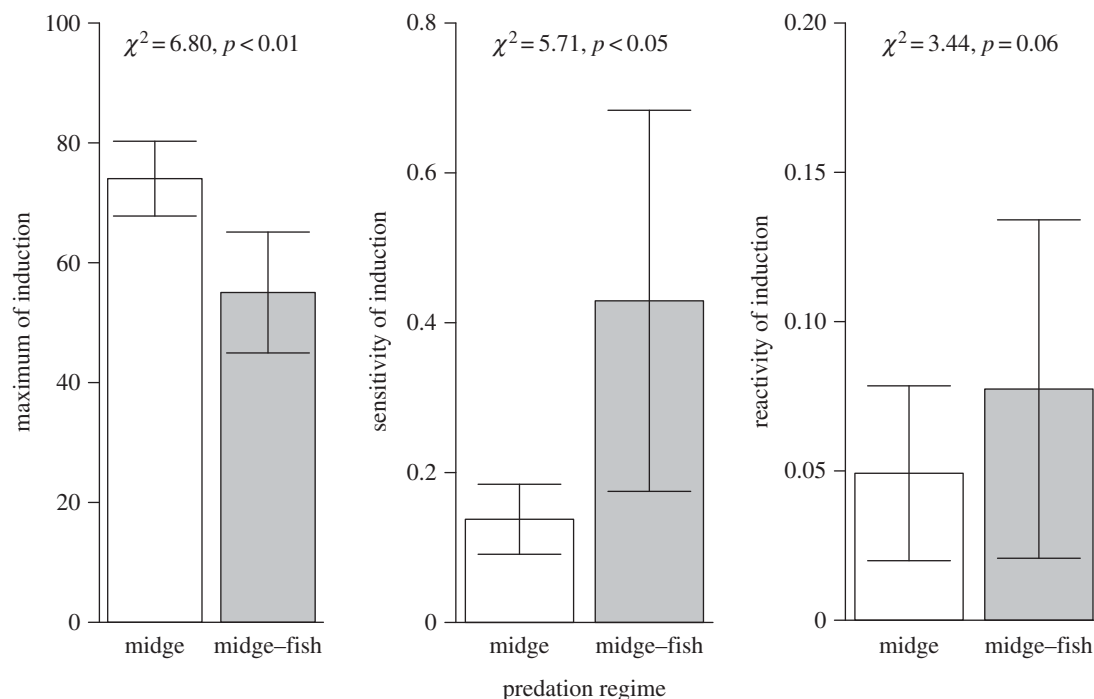
Figure 2. Parameter mean $\pm$ s.e. values that describe the neckteeth reaction norms to Bagshaw (midge) and Crabtree (fish−midge) populations of *Daphnia pulex*. Statistical comparison was performed with maximum likelihood analysis treating predation regime as fixed effect.

Table 2. G and P (co)variance matrices estimates from the pooled population (midge/fish−midge as fixed effect). The parameters correspond to mode of posterior distribution from data that were standardized to mean = 0 and s.d. = 1 prior to estimating variance components. The significant genetic parameters are indicated by (*), after comparing DIC (electronic supplementary material, table S2) between models.

	maximum (IC)	sensitivity (IC)	reactivity (IC)
G-matrix			
maximum	0.18 (0.03; 0.53)*	−0.12 (−1.54; −0.18)*	−0.001 (−0.72; 0.09)
sensitivity		0.35 (0.12; 0.95)*	0.03 (−0.09; 0.79)
reactivity			0.02 (0.005; 0.43)
P-matrix			
maximum	0.36 (0.23; 0.61)	−0.13 (−0.28; 0.45)	−0.07 (−0.30; 0.12)
sensitivity		0.53 (0.32; 0.85)	0.20 (0.03; 0.53)
reactivity			0.79 (0.53; 1.36)

(indirect) selection responses [46]:

$$\Delta \bar{Z}_i \text{ (direct)} = G_i \beta_i \quad \text{and} \quad \Delta \bar{Z}_{i,j} \text{ (indirect)} = \sum_{i \neq j} G_i \beta_i. \quad (2.2a,b)$$

We examined whether any changes in genetic (co)variation can be ascribed to nonlinear and correlated selection (γ—the matrix of nonlinear selection gradient) [36].

3. Results

(a) Reaction norms

We found significant genetic and population specific variation in the maximum (asymptote) and sensitivity (threshold/ inflection) of the reaction norms (figures 1 and 2). Genotypes sourced from the midge pond (Bagshaw) were characterized by high levels of induction (74.0 ± 6.24 induction) and a sensitive (closer to 0) threshold (0.13 ± 0.04 µl ml^{-1}). Genotypes sourced from the fish−midge pond (Crabtree) show comparatively lower levels of induction (55.0 ± 10.1 induction) and a

less sensitive threshold (0.42 ± 0.25 µl ml^{-1}). A small-scale parameter (reactivity) in both ponds suggests a steep, threshold-like response (midge: 0.05 ± 0.02; fish: 0.08 ± 0.05). Our fitness proxy, defined as the log quotient of age at maturity and first clutch size, did not differ between ponds ($\chi^2 = 1.48$, $p = 0.22$).

(b) G-matrix

We found significant genetic variation in the maximum amount of induction (asymptote) and the sensitivity (threshold/inflection) to cues (table 2), with genetic variation in the sensitivity being lower than in the maximum. We also found a negative and significant genetic covariance between the maximum and sensitivity of induction (table 2). This represents a positive covariance in 'biological' terms as reducing the sensitivity (more sensitive to kairomones) and increasing the maximum response are both deemed 'beneficial' in the face of midge predation. Thus, this G-matrix, comprised of individuals from two populations, reflects a potentially

Table 3. Summary of linear and quadratic selection analyses and M matrix of eigenvectors from canonical analysis of γ_{matrix} for reaction norms parameters of both populations. The coefficients were obtained from parameters (maximum, sensitivity and reactivity) that describe the reaction norm of sigmoid fit of neckteeth *D. pulex*. Standardized directional selection coefficient (β), standardized nonlinear and correlated selection coefficients (γ_{matrix}). The nonlinear coefficients reported were doubled from the originals (in parenthesis) following the suggestion of Stinchcombe *et al.* [42]. Significant coefficients were obtained by randomization (10 000) test.

traits	β	γ maximum	sensitivity	reactivity	λ_i	M matrix maximum	sensitivity	reactivity
midge (Bagshaw)								
maximum	0.029	0.189 (0.09)*			0.227	0.581	0.779	0.231
sensitivity	0.016*	−0.175	0.095 (0.047)		0.035*	−0.616	0.236	0.751
reactivity	0.008	0.087	−0.225	0.096 (0.05)	−0.072	0.531	−0.579	0.618
fish−midge (Crabtree)								
maximum	0.009	0.051 (0.03)			0.121**	0.259	0.844	0.468
sensitivity	−0.018*	0.008	0.202 (0.10)*		0.048**	0.898	−0.388	0.204
reactivity	0.008	−0.116	−0.091	−0.073 (−0.04)	−0.415	−0.354	−0.367	0.859

*$p < 0.05$; **$p < 0.01$.

strong constraint where the maximum induction (asymptote) and the sensitivity to predator cues (threshold/inflection) respond together in a biologically meaningful manner.

(c) Selection gradients

The selection gradient estimates for our fitness proxy in each predation regime were distinct. Midge pond data revealed directional (linear) and nonlinear (disruptive) selection on maximum induction (table 3). Fish−midge pond data revealed nonlinear (disruptive) selection on the sensitivity of induction (table 3).

The canonical analysis revealed a significant and positive eigenvalue among traits for the midge pond. The eigenvector associated with the dominant eigenvalue was characterized by opposite loadings for the maximum and the sensitivity of induction, paralleling the patterns of selection indicated on the original axis (table 3). The canonical analysis for data from fish−midge pond revealed no significant eigenvalues. The first eigenvalue was linked to an eigenvector dominated by the threshold weighting, again paralleling the patterns on the original scale (table 3). The canonical analyses for each predation scenarios produced a set of three eigenvalues with different sign, suggesting that the overall selection surface is a saddle [42]. We note the absence of selection pressure on the reactivity (slope) of the response, where we also found no significant genetic variation.

(d) The micro-evolution of a predator-induced plasticity

Table 4 and figure 3 highlight the predicted response to selection of the reaction norm of induced morphological defence. Figure 3a captures the response of each population reaction norm assuming that only the reproduction component of the selection gradient matters. Selection driven only by reproduction drives convergence of the environment-specific reaction norms towards a maximum and sensitivity that is in between that defined by a midge or a fish−midge environment.

Figure 3b shows the result of applying a hypothetical survival-only selection gradient to the components of the reaction norm. Here we see rather subtle, but theoretically expected shifts, where the maximum defence is elevated in both environments, but the midge regime sees increased sensitivity to the cue (threshold moves left), while the fish−midge regime sees decreased sensitivity (threshold moves right).

Finally, figure 3c shows the effect of equally weighted but combined response to selection on reproduction and survival. Here we see the consequences of combining both selection gradients and the constraint represented by the negative covariation between the maximum and the sensitivity in the G-matrix. The midge regime experiences a small decrease in maximum induction and less sensitivity to the cue, completely at odds with what theory might predict. By contrast, the fish−midge regime sees not only decreased sensitivity (as would be expected), but also increased maximum, which may or may not be expected (see below).

4. Discussion

Predator-induced defences are one of the most studied examples of phenotypic plasticity [2,18]. This research is grounded in a body of well-developed theory and empirical work spanning decades, debates and taxa [47,48] with daphnids forming a core body of this work. Empirical, evolutionary ecological research with daphnids is broad and has revealed fascinating insight into plasticity in the life history [49,50], behaviour [51,52], morphology [8,19,49] and costs of plasticity [8,26,49,53−55]. Recent work as revealed detail about $G \times E$ in, and reaction norms of, morphological and other defences [8,25,56]. Despite this breadth of work, no evidence about the potential micro-evolution of morphological reaction norms has been reported. Therefore, we explored how a predator-induced, nonlinear, reaction norm of induced morphological defence might respond evolutionarily to contrasting size-selective predation regimes.

Our method, developed in the context of *D. pulex* ecology, involved decomposing a nonlinear, predator-induced reaction norm to three biologically relevant traits. This showed that it is possible to analyse the micro-evolution of these nonlinear reaction norms with well established, character-state tools. Our data provided a fine scale description of the reaction

Table 4. The response to selection (ΔZ) using composite selection gradients based upon different weighting of reproduction (β_R) and survival (β_S) selection. The multivariate response to selection is partitioned into trait-specific direct (through genetic variance), indirect (through genetic covariance) and total (using all components of G) response in the fish and midge cue treatment.

	trait	total response	direct selection	indirect selection
midge pond response				
$\Delta Z_{\beta(R)}$	maximum	-0.089 (-0.254; -0.039)[a]	0	-0.089 (-0.254; -0.039)[a]
$\Delta Z_{\beta(R)+0.5\beta(S)}$	maximum	0.393 (0.168; 1.134)[a]	0.341 (0.146; 0.884)[a]	-0.119 (-0.045; 0.378)
$\Delta Z_{\beta(R)+\beta(S)}$	maximum	0.889 (0.451; 2.292)[a]	0.680 (0.270; 1.523)[a]	0.230 (-0.039; 0.872)
$\Delta Z_{0.5\beta(R)+\beta(S)}$	maximum	0.942 (0.478; 2.416)[a]	0.680 (0.270; 1.523)[a]	0.280 (0.017; 0.997)[a]
$\Delta Z_{\beta(S)}$	maximum	0.995 (0.500; 2.551)[a]	0.68 (0.27; 1.523)[a]	0.344 (0.071; 1.113)[a]
$\Delta Z_{\beta(R)}$	sensitivity	0.121 (0.062; 0.322)[a]	0.121 (0.062; 0.322)[a]	0
$\Delta Z_{\beta(R)+0.5\beta(S)}$	sensitivity	-0.364 (-1.099; -1.152)[a]	-0.214 (-0.627; -0.092)[a]	-0.165 (-0.598; 0.032)
$\Delta Z_{\beta(R)+\beta(S)}$	sensitivity	-0.993 (-2.405; -0.404)[a]	-0.585 (-1.562; -0.301)[a]	-0.271 (-1.128; -0.037)[a]
$\Delta Z_{0.5\beta(R)+\beta(S)}$	sensitivity	-1.222 (-2.582; -0.449)[a]	-0.645 (-1.723; -0.332)[a]	-0.271 (-1.128; -0.037)[a]
$\Delta Z_{\beta(S)}$	sensitivity	-1.101 (-2.772; -0.503)[a]	-0.706 (-1.885; -0.363)[a]	-0.271 (-1.128; -0.037)[a]
$\Delta Z_{\beta(R)}$	reactivity	0.039 (-0.013; 0.147)	0	0.039 (-0.013; 0.147)
$\Delta Z_{\beta(R)+0.5\beta(S)}$	reactivity	-0.125 (-0.420; 0.047)	0.014 (0.005; 0.237)[a]	-0.213 (-0.628; 0.05)
$\Delta Z_{\beta(R)+\beta(S)}$	reactivity	-0.300 (-0.934; 0.160)	0.059 (0.006; 0.474)[a]	-0.398 (-1.388; 0.088)
$\Delta Z_{0.5\beta(R)+\beta(S)}$	reactivity	-0.315 (-1.003; 0.166)	0.059 (0.006; 0.474)[a]	-0.384 (-1.490; 0.135)
$\Delta Z_{\beta(S)}$	reactivity	-0.330 (-1.080; 0.153)	0.059 (0.006; 0.474)[a]	-0.400 (-1.575; 0.125)
fish$-$midge response				
$\Delta Z_{\beta(R)}$	maximum	0.009 (0.004; 0.027)[a]	0	0.009 (0.004; 0.027)[a]
$\Delta Z_{\beta(R)+0.5\beta(S)}$	maximum	-0.590 (-1.587; -0.255)[a]	-0.364 (-0.884; -0.146)[a]	-0.221 (-0.754; -0.098)[a]
$\Delta Z_{\beta(R)+\beta(S)}$	maximum	-1.274 (-2.974; -0.531)[a]	-0.680 (-1.523; -0.270)[a]	-0.510 (-1.459; -0.226)[a]
$\Delta Z_{0.5\beta(R)+\beta(S)}$	maximum	-1.280 (-2.986; -0.533)[a]	-0.680 (-1.523; -0.270)[a]	-0.514 (-1.472; -0.228)[a]
$\Delta Z_{\beta(S)}$	maximum	-1.286 (-2.997; -0.536)[a]	-0.680 (-1.523; -0.270)[a]	-0.519 (-1.486; -0.230)[a]
$\Delta Z_{\beta(R)}$	sensitivity	-0.013 (-0.034; -0.007)[a]	-0.013 (-0.034; -0.007)[a]	0
$\Delta Z_{\beta(R)+0.5\beta(S)}$	sensitivity	0.646 (0.272; 1.682)[a]	0.314 (0.135; 0.919)[a]	0.229 (0.101; 0.783)[a]
$\Delta Z_{\beta(R)+\beta(S)}$	sensitivity	1.348 (0.668; 3.215)[a]	0.693 (0.357; 1.850)[a]	0.519 (0.230; 1.486)[a]
$\Delta Z_{0.5\beta(R)+\beta(S)}$	sensitivity	1.354 (0.671; 3.230)[a]	0.699 (0.360; 1.867)[a]	0.519 (0.230; 1.486)[a]
$\Delta Z_{\beta(S)}$	sensitivity	1.361 (0.674; 3.246)[a]	0.706 (0.363; 1.885)[a]	0.519 (0.230; 1.486)[a]
$\Delta Z_{\beta(R)}$	reactivity	-0.004 (-0.016; 0.001)	0	-0.004 (-0.016; 0.001)
$\Delta Z_{\beta(R)+0.5\beta(S)}$	reactivity	0.115 (-0.047; 0.75)	0	-0.115 (-0.047; 0.751)
$\Delta Z_{\beta(R)+\beta(S)}$	reactivity	0.397 (-0.123; 1.561)	0	0.397 (-0.123; 1.561)
$\Delta Z_{0.5\beta(R)+\beta(S)}$	reactivity	0.399 (-0.124; 1.568)	0	0.399 (-0.124; 1.568)
$\Delta Z_{\beta(S)}$	reactivity	0.400 (-0.125; 1.575)	0	0.400 (-0.125; 1.575)

[a]Posterior mode and the upper and lower bound of the 95% credibility interval are presented, and elements significantly different from zero.

norms and show that genetic variation might be predator-regime and population specific. Critically, by analysing the response of the reaction norm to plausible, predator-specific selection gradients, we found that a G-matrix, harbouring a strong covariance between the sensitivity to cues—the threshold part of this threshold trait—and the maximum response to cues, predicted a rather more uniform morphological response to predation risk than might be expected under size-selective predation theory about morphological defences [2].

Our results formalize a hypothesis that selection on survival and reproduction combine with covariance between the maximum morphological response to these cues (asymptote) and the sensitivity of morphology (threshold/inflection) to midge predation cues to shape the micro-evolutionary trajectory of predator-induced defences in *D. pulex*. This covariance between the two parts of the defence captures patterns of variation seen in figure 1 among the genotypes and is a major influence on our simulation of micro-evolutionary change.

(a) Evolution of a reaction norm

We began our assessment of micro-evolutionary change to a nonlinear reaction norm with data showing that the maximum response (asymptote) and the sensitivity to the cue

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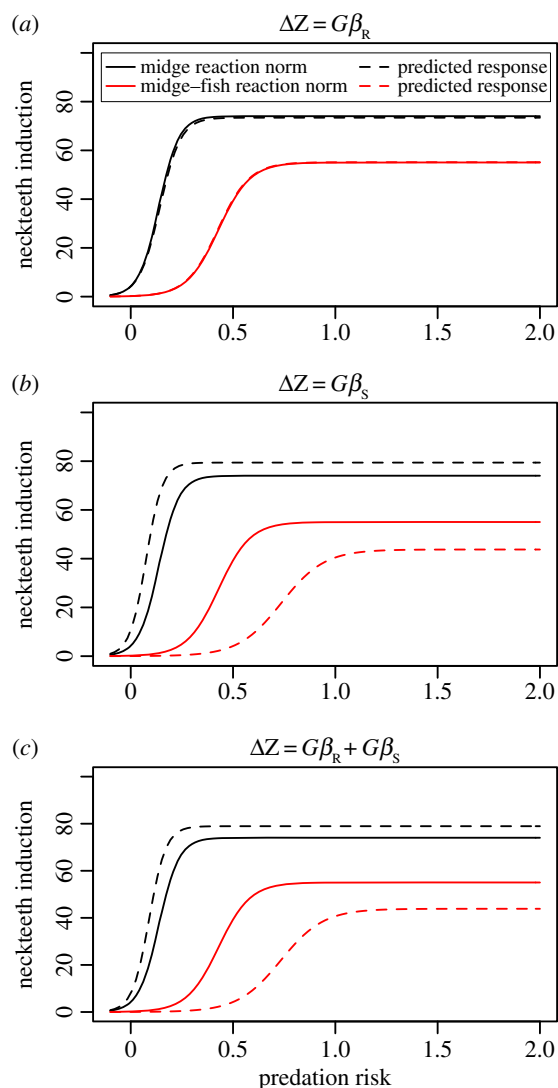
Figure 3. Projected neckteeth reaction norm under midge and fish predation scenarios considering the reproduction component of selection gradient (*a*), the survival component of selection gradient (*b*) and the combined reproduction and survival selection gradient (*c*). Differences in magnitude and orientation of linear selection gradient β and response to selection Δz. (Online version in colour.)

(threshold/inflection) harbour significant genetic variation (figure 1 and table 2). The data suggest that the midge population harbours little variation in sensitivity and a fair amount in the maximum induction while the fish–midge population harbours larger variation in the sensitivity, and less in the maximum. While sample sizes are low, these data provide a template on which to explore hypotheses about micro-evolutionary change in reaction norms. Importantly, we found in the combined data a negative genetic correlation between the maximum response and sensitivity to the cue, defining a biologically meaningful constraint (not trade-off) of increased sensitivity (reducing threshold) and increased induced morphology (increasing maximum). We highlight that our sigmoid model of the reaction norm specifies three potential traits, motivated by capturing variation visible in figure 1 through our experimental design with several maternal lines. While such a model has been used often in work on threshold traits, the approach and insights are not defined by the model, but by the capacity to capture meaningful levels of genetic (co)variation associated with the parameters.

We considered several ways in which selection might act on this genetic variation [41], combining empirical data on reproduction linked to variation in defence traits with size-selective theory about patterns of survival linked to the same traits. Our composite selection scenarios represent several weightings of selection linked to survival and reproduction to which a population may respond. This was combined in the multivariate breeders equation with the constraints explicit in the *G*-matrix (figure 3). Our results reveal not only the potential for substantially different response to selection pattern depending on the predation risk scenario, but also how strong covariation between traits might constrain the response.

Although our analysis is developed from data from only two populations, we argue cautiously about the effect of selection on population differentiation. However, previous evidence about cost of plasticity [8] and differences in phenotypic trajectories [25] support this possibility in relation to realistic patterns of the evolution of the reaction norm.

(b) Covariance matters

As noted above, we found a biologically meaningful negative correlation between maximum response and sensitivity to cue that represents a potential evolutionary constraint. Were this to be an accurate representation of the three traits in a larger pool of populations, combining the selection gradients with this *G*-matrix provides an interesting perspective on how evolution might act on predator-induced plasticity. When the total response of selection is decomposed to its direct and indirect effect, the relevance of the genetic covariance became clear. The significant and highly negative genetic covariance between maximum and threshold constrained the response of selection in the two different predation scenarios. The relatively large angle between β's and ΔZ's reinforced this view, because the covariance resulted in a response to selection significantly different from the direction of selection. They were similar between midge (Bagshaw) and fish–midge (Crabtree) predation scenarios, reflecting the covariance pattern.

Thus, the indirect response of selection seems to play a crucial role in the total response of selection, driving similar patterns of phenotypic response from different predation scenarios [57]. If the patterns of multivariate genetic variation we find is common, the constraint may represent an important genetic pathway underpinning local adaptation in daphnids facing multiple and variable regimes of predation risk [10,26,51,58,59]. Our data suggest that the evolution of midge-induced morphological plasticity is robust to the presence of an alternate, contrasting selection pressure that does not induce the morphological change.

Although our *G*-matrix is based only on morphological trait data from only two populations, our results align with the theory about size-selective predation [20] and thus represent a plausible empirical parametrization of theory and the potential outcome of selection in natural environments. It allowed us to generate a hypothesis about ecologically driven micro-evolution of the predator-induced reaction norm of the morphological defence. It also provides a template on which one can apply theory about reaction norm evolution under more extreme selection [60].

The role of phenotypic plasticity in the process of adaption is still a topic pushing theory and discussion [27,48,61]. However, several lines of evidence suggest that phenotypic plasticity may be an important driver of evolutionary change in heterogeneous

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environments [4,62–64]. Crucial to these arguments is that plasticity might facilitate the maintenance of genetic variation in populations that inhabit heterogeneous environments. Phenotypic plasticity is thus a mechanism that may aid the persistence of populations, allowing for higher rates of adaptation [27,61,65–68]. Empirical evidence related to the ability of organisms to evolve in response to biotic, agonistic interactions [69–71] suggest that this link between plasticity and evolvability is real. Our data add to this knowledge, showing how fine scale, multivariate predator-induced phenotypic plasticity likely influences the outcome of evolution in heterogeneous predator environments.

5. Conclusion

Predation is a key selective agent in aquatic and terrestrial communities. Seasonal and spatial variation in predation risk has favoured the evolution of inducible defences, a major example of phenotypic plasticity. The response of traits to predation risk is rarely effectively characterized by a linear reaction norm describing plasticity. Characterizing genetic variation and covariation in a nonlinear relationship is not trivial, but it is vital for understanding how agents of natural selection such as predators drive the evolution of

phenotypic plasticity. Using a model system of daphnids facing predation risk from multiple predators, we show, empirically, one route to describing variation in and micro-evolutionary response of a nonlinear reaction norms. Our representation of genetic (co)variance in the reaction norm allowed us to explore the potential micro-evolutionary response of the reaction norm shaped by variable predation risk and constraints on the evolution of specific features of the reaction norm.

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