

Phenotypic plasticity facilitates recurrent rapid adaptation to introduced predators

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A central role for phenotypic plasticity in adaptive evolution is often posited yet lacks empirical support. Selection for the stable production of an induced phenotype is hypothesized to modify the regulation of preexisting developmental pathways, producing rapid adaptive change. We examined the role of plasticity in rapid adaptation of the zooplankton *Daphnia melanica* to novel fish predators. Here we show that plastic up-regulation of the arthropod melanin gene dopa decarboxylase (*Ddc*) in the absence of UV radiation is associated with reduced pigmentation in *D. melanica*. *Daphnia* populations coexisting with recently introduced fish exhibit environmentally invariant up-regulation of *Ddc*, accompanied by constitutive up-regulation of the interacting arthropod melanin gene *ebony*. Both changes in regulation are associated with adaptive reduction in the plasticity and mean expression of melanin. Our results provide evidence that the developmental mechanism underlying ancestral plasticity in response to an environmental factor has been repeatedly co-opted to facilitate rapid adaptation to an introduced predator.

genetic accommodation | genetic assimilation | gene expression | pigmentation | rapid evolution

The claim that phenotypic plasticity can facilitate adaptation remains controversial following a century of investigation (1–5). According to modern proponents, genetic accommodation (genetic change in the regulation or form of a plastic trait) can channel and accelerate evolutionary divergence (6, 7). This argument centers on the fact that plastic organisms possess the developmental mechanism to form an alternative, induced phenotype. Selection for the stable production of this phenotype can act on regulation of preexisting developmental pathways to effect adaptive change. One hypothesized result is recurrent rapid evolution in the direction of ancestral plasticity (6–9), based on changes in the expression, rather than the structure, of genes (10). A number of artificial selection experiments demonstrate rapid genetic accommodation within lineages (11–13), providing support for the plausibility of this phenomenon in nature. In addition, many studies of natural populations reveal congruent patterns of plasticity and adaptive change at the population and species level (8, 9, 14). However, the importance of this process remains difficult to assess, due to three factors: it is usually impossible to observe ancestral reaction norms (but see ref. 14); it is difficult to elucidate the developmental details of both plasticity and adaptation; and adaptation through genetic accommodation is thought to proceed rapidly, making it likely that researchers will observe the results of this process rather than capture it in action (8). As a consequence, there is little to no empirical evidence for a significant role of plasticity in facilitating adaptive evolution in natural populations (3–5, 15).

In the high UV environment of Sierra Nevada alpine lakes (California), the microcrustacean *Daphnia melanica* is normally darkly pigmented and thus highly visible. Salmonid fish (*Onchorhynchus*, *Salmo*, and *Salvelinus* spp.), which are visual predators of *Daphnia*, were introduced into many of these historically fishless lakes during the last century, resulting in either local extinction of *D. melanica* (16) or recurrent, rapid adaptation in a suite of morphological and life-history traits (17). Consistent with

known patterns of selection pressure from fish predation (18), populations of *D. melanica* that coexist with introduced fish exhibit reduced pigmentation compared to populations without fish predators. We characterized the UV-related reaction norm of melanin in 13–17 randomly selected genotypes from two lakes that have never experienced fish predation and two lakes that have supported populations of fish for 53 and 91 years, respectively. To accomplish this, we measured melanin levels in 1–3 replicate clones of each genotype raised under UV and non-UV laboratory conditions. We also measured clutch size, body size, maturation time, and percent mortality for each genotype in each environment to evaluate the adaptive value of ancestral plasticity. Finally, to gain insight into the mechanisms underlying plasticity and adaptation, we measured levels of expression for homologs of two genes known to be involved in arthropod melanization: dopa decarboxylase (*Ddc*) and *ebony* (19).

Results and Discussion

Our melanin assay revealed a pattern of adaptation through genetic accommodation (Fig. 1*A* and *D*; means and standard errors of all traits are detailed in Table S1). Melanin was significantly plastic with respect to UV in the ancestral state (fishless) populations (ANOVA on log-transformed data, $F_{1,65} = 84.99$, $P < 0.0005$). This plasticity appears to be adaptive: melanin improved survival in UV environments, but incurred a cost in non-UV environments by slowing maturation (SI Text). Individual *Daphnia* can modulate their level of melanin each molt (typically every 3 days at 18°C), and seasonal variation in UV intensity, as well as the tendency for *Daphnia* to migrate downward in the water column in response to both fish predation and UV exposure (20), would logically expose the induced (i.e., lower melanin) phenotype to selection. Populations that coexist with fish exhibited reduction of plasticity in melanin, as evidenced by a significant UV by fish history interaction, as well as reduction in mean levels of melanin, as evidenced by a significant effect of fish history (Table 1 and Fig. 1*D*). Both modifications to the reaction norm serve to reduce deposition of melanin under natural, high-UV conditions. Genetic data indicate that our populations with fish have independent evolutionary trajectories (SI Text, Figs. S1 and S2, and Tables S2 and S3). Coupled with previous studies of fish predation on melanic *Daphnia* (18), the recurrent nature of change in populations with fish strongly suggests that this reduction in melanin is adaptive (21).

Examination of transcriptional regulation of the arthropod pigmentation pathway genes *Ddc* and *ebony* revealed strong associations with melanin production. We used a general linear model (GLM) to predict melanin as a function of population nested within fish history, fish history, and UV treatment (i.e., all significant main

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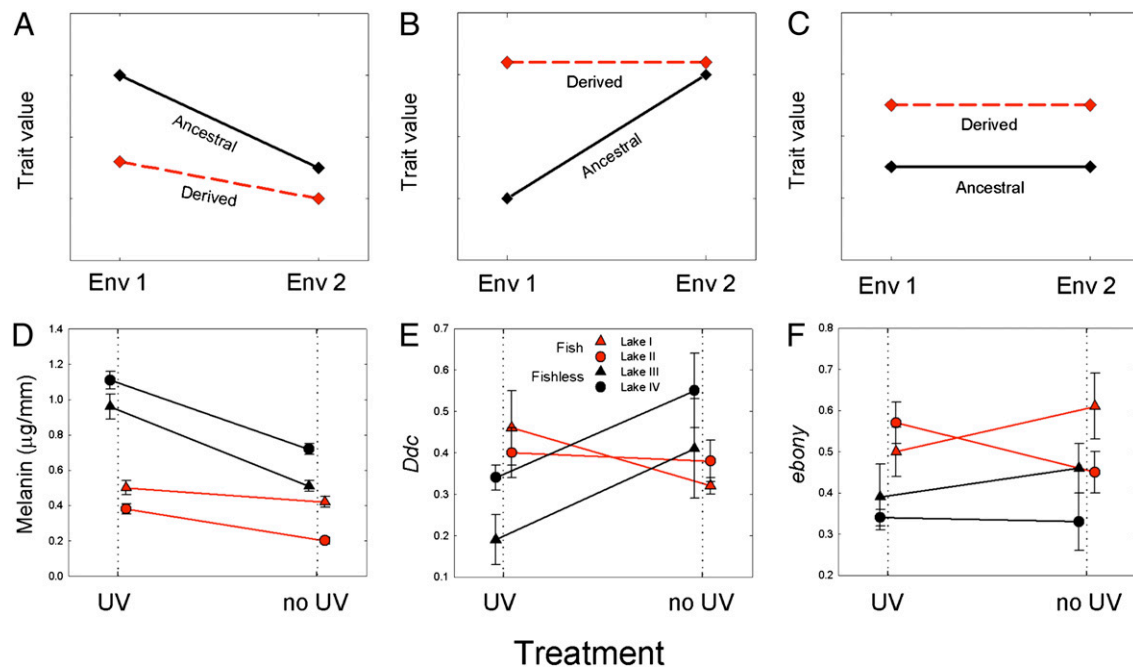


Fig. 1. Evolution of reaction norms. Three theoretical patterns are illustrated. (A) One common form of genetic accommodation: reduction of the magnitude and environmental sensitivity of a trait; (B) genetic assimilation; and (C) constitutive up-regulation. Our results show (D) genetic accommodation in melanin, (E) loss of plasticity in *Ddc* expression, and (F) constitutive up-regulation in *ebony* expression. Gene expression is a relative measure, reported in terms of the highest expression level among samples. Data points are laterally offset to aid visualization. Error bars represent a single standard error. Lake I is Puppet, lake II is Evelyn, lake III is Source, and lake IV is Frog.

effects, as evaluated by ANOVA; Table 1), plus expression of *Ddc*, *ebony*, and the interaction between these two genes. This analysis provides a conservative test of the association between gene expression and melanin by evaluating only residual variance in melanin. Our results revealed a significant effect of *Ddc*, *ebony*, and *Ddc* by *ebony* interaction (Table 2). The association between gene expression and melanin production is visualized via construction of a phenotypic landscape (22), in which the expected level of melanin is plotted as a function of *Ddc* and *ebony* expression (Fig. 2). The significant nonadditive interaction between these two genes creates curvature in the surface of this landscape (22).

At the mean level of *Ddc* expressed by ancestral-state populations (i.e., fishless populations exposed to UV), up-regulation of *ebony* is associated with reduction in melanin (Table 2 and Fig. 2). This pattern is consistent with previous studies of *ebony* and likely results from the role of the *ebony* protein in converting dopamine into N- β -alanyl dopamine sclerotin rather than dopamine melanin (19). Similarly, at the mean level of *ebony* expressed by ancestral-state populations, up-regulation of *Ddc* is associated with reduc-

tion in melanin (Table 2 and Fig. 2). In insects, *Ddc* converts DOPA into dopamine in the melanin pathway (19), and early up-regulation of *Ddc* is associated with a developmental switch away from black pigment (23). However, because the dopamine precursor for dopamine melanin can come either directly from DOPA (via the gene *yellow*) or from N- β -alanyl dopamine (via the gene *tan*) (19, 24), the relationship between *Ddc* regulation and melanin production is complex and dependent upon regulation in additional genes in the dopamine pathway. The significant interaction between *ebony* and *Ddc* is thus unsurprising, although the mechanism of this interaction requires further study and is likely dependent upon expression in other genes, such as *yellow*, *tan*, or *aaNAT* homologs (see ref 19).

Patterns of expression in *Ddc* and *ebony* are associated with both plasticity and adaptation. A significant fish history by UV interaction indicates evolutionary loss of plasticity in *Ddc* expression with exposure to fish predation (Table 1 and Fig. 1E). An ANOVA on log-transformed expression shows that ancestral state (fishless) populations exhibit significant plasticity in *Ddc* expression with respect to UV ($F_{1,21} = 7.60$, $P = 0.01$) but populations with fish do

Table 1. ANOVA results for melanin and gene expression

Effect	Melanin			<i>Ddc</i>			<i>ebony</i>		
	df	F	P	df	F	P	df	F	P
Clone	48	1.21	0.199	20	2.36	0.026	20	1.44	0.203
Population	2	15.37	0.000	2	1.35	0.281	2	0.97	0.395
Fish	1	180.46	0.000	1	0.07	0.798	1	9.44	0.006
UV	1	92.31	0.000	1	3.00	0.097	1	0.09	0.767
UV x fish	1	27.72	0.000	1	13.19	0.001	1	0.25	0.621

Each response variable was modeled as a function of clone nested within population, population nested within fish history, fish history, UV treatment, and fish history by UV treatment interaction, with clone as a random effect. Significant results ($P < 0.05$) are indicated in bold. Melanin data include replicates within clones; the error df = 121 and total df = 174. For gene expression, error df = 22 and total df = 47.

Table 2. General linear model results

Effect	Coefficient	df	F	P
Pop		2	9.99	0.000
Fish		1	86.09	0.000
UV		1	28.14	0.000
<i>ebony</i>	-1.31	1	9.30	0.004
<i>Ddc</i>	-1.66	1	12.20	0.001
<i>Ddc</i> x <i>ebony</i>	3.02	1	8.54	0.006

Melanin is modeled as a function of population nested within fish history, fish history, UV treatment, and expression of *ebony*, *Ddc*, and *ebony* by *Ddc* interaction. Significant results ($P < 0.05$) are indicated in bold. The error df = 39 and total df = 46.

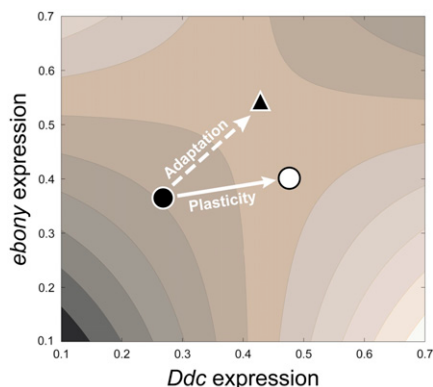


Fig. 2. Contour map of expected level of melanin as a function of *ebony* and *Ddc* expression. Darker contours indicate greater melanin deposition. Mean levels of gene expression are indicated for fishless populations (circles) and populations with fish (triangle), reported in terms of the highest expression level among samples. Fill color indicates treatment with UV (black) or no UV (white). The solid arrow represents a plastic developmental shift in fishless populations. The dotted arrow represents an adaptive shift due to fish predation. Populations with fish do not significantly change when UV is absent (Table 1).

not ($F_{1,21} = 0.80$, $P = 0.38$). In addition, expression of *Ddc* is significantly up-regulated in populations with fish when measured under UV conditions ($F_{1,20} = 7.20$, $P = 0.01$), but is not significantly different between fish and fishless populations when UV light is absent ($F_{1,20} = 1.26$, $P = 0.28$). In contrast, *ebony* is invariant with respect to UV but constitutively up-regulated in populations with fish (Table 1 and Fig. 1 C and F). The pattern of *Ddc* expression is akin to genetic assimilation (2) in the sense that up-regulation (compared to fishless populations in UV conditions) is environmentally induced in fishless populations but invariant in populations with fish (Fig. 1 B and E). However, expression of *Ddc* does not show unambiguous fixation at the ancestral-induced level (i.e., the level of fishless populations in no-UV conditions). Instead, average *Ddc* expression in fish populations remains invariant at a level expected to generate less melanin, given their increased average level of *ebony* expression (Fig. 2). For both genes, the lack of any significant population-level effect suggests that fishless populations do not differ from one another, and that populations with fish have evolved the same changes in gene expression (Table 1).

The evolutionary importance of genetic accommodation depends on the tendency for preexisting plasticity to provide the developmental mechanism for adaptation. It is thus critical to assess the relationship between ancestral plasticity and derived change at the developmental level. The phenotypic landscape for melanin provides a succinct means to visualize this relationship (22). For fishless populations, plotting the mean level of expression of *Ddc* and *ebony* under UV and non-UV conditions illustrates plastic developmental change that moves the population downhill on the phenotypic landscape (i.e., results in reduced levels of expected melanin) (Fig. 2). Plotting the mean level of expression of *Ddc* and *ebony* in ancestral (fishless) populations and derived (fish) populations, both under realistic (i.e., UV) conditions, illustrates adaptive developmental change. This change in gene expression also moves the population downhill on the phenotypic landscape (Fig. 2). In this case, the same developmental mechanism (*Ddc* up-regulation) is associated with both plastic and adaptive change. Additional up-regulation of *ebony* in populations with fish causes the vector of adaptive change to be aligned with the gradient of steepest descent. In addition, it reduces the degree to which *Ddc* up-regulation will translate into higher levels of melanin (Fig. 2). This provides evidence that evolution of expression in *ebony* acted in concert with

evolution of the *Ddc* reaction norm to increase the rate of evolutionary reduction in melanin.

Evolution via genetic accommodation has been invoked to explain the parallel nature of adaptive radiation in numerous systems, including the repeated evolution of specific ecotypes in sticklebacks (14), *Anolis* lizards (25), and Australian tiger snakes (26). Ancestral plasticity is thought to provide the developmental mechanism for shifts between ecotypes, explaining the rapid and recurrent emergence of particular forms (6, 9). This hypothesis contrasts with studies that demonstrate parallel evolution based on co-option of ancestral alleles in genes of major effect (27) or change in *cis*-regulatory elements of developmental control genes not involved in ancestral plasticity (28–30). In this case, we provide evidence that ancestral plastic up-regulation of *Ddc* has been repeatedly co-opted to produce rapid adaptation in melanin, in concert with evolution of expression in the interacting gene *ebony*. *Ddc* is known to play a role in a wide variety of functions, including the production of neural transmitters, wound healing, parasite defense, behavior, and cuticle hardening (31). The involvement of *Ddc* in ancestral plasticity indicates a preexisting developmental architecture that allows for modulation without detrimental pleiotropic effects—a pattern that would logically facilitate rapid evolution of expression. In general, the concept of evolution via genetic accommodation shows how selection may frequently relate to changes in regulation and is congruent with a growing recognition of the importance of regulatory changes in the process of adaptation (32). Finally, because adaptation has proceeded over just 53–91 years of exposure to fish predation, a factor often resulting in local extinctions within this system (16), our results highlight the role of genetic accommodation in facilitating rapid evolutionary responses that have potentially profound ecological implications.

Methods

Clone Maintenance. We collected individual genotypes from two permanent lakes that retain their naturally fishless condition (Frog and Source) and two permanent lakes that have been stocked with golden trout (*Oncorhynchus mykiss aguabonita*) since 1951 (Puppet) or brown trout (*Salmo trutta*), brook trout (*Salvelinus fontinalis*), and rainbow trout (*Oncorhynchus mykiss*), beginning in 1913 (Evelyn). Lake locations, stocking information, and procedures for the collection and maintenance of clones are detailed in ref. 17.

We assayed all traits using modification of a standard experimental design (33). After holding two generations in strictly controlled conditions, we placed the third (experimental) generation into separate beakers on the day after birth and randomly assigned these beakers to either control or UV conditions. Control beakers were placed under standard fluorescent lights as well as plastic Lumar screens that block all UV radiation. UV treatment beakers were placed 10.5 cm below 30 W, 36-inch Reptisun 5.0 UVB fluorescent lightbulbs (Zoo Med), and fans were used to ensure that UV treatment and control beakers retained the same 18 °C temperature. The dose of UVB from these lamps was roughly 10% that at the lake surface during summer solstice, or equivalent to what *Daphnia* would experience ≈ 9 m below the water surface, according to estimates of UVB dose (27.9 W · h/m²) and dissolved organic carbon attenuation coefficient (0.26) obtained from nearby Sequoia National Park (34). Opaque screens separated control and UV beakers. Throughout the experiment, we maintained all *Daphnia* in the same controlled temperature room at 18 °C with a 16L:8D photoperiod, in 200 mL of filtered well-water that was supplemented with a constant concentration (135,000 cells/mL) of the green algae *Scenedesmus obliquus* and replaced every other day.

For the experimental generation, we recorded the time from birth to maturity for the fastest-maturing member of each clutch, defining maturity as the first instar with deposition of eggs into the brood pouch. We also recorded the number of clutch failures (i.e., clutches in which all individuals died before maturity). When the first clutch of eggs from experimental generation individuals had developed enough to produce two separate eyes (i.e., just before adult molting), we randomly assigned these individuals to preservation for either melanin or RNA extraction. Preservation only at this stage reduced variance, given that *Daphnia* deposit melanin throughout each instar and become darkest just before molting. Individuals assigned to melanin extraction were measured for length and placed into 4% formalin.

Individuals assigned to RNA extraction were placed into RNeasy (Qiagen). In both cases, *Daphnia* samples were stored at -20°C until extraction.

Melanin Assay. We incubated *Daphnia* preserved for melanin extraction in $100\ \mu\text{L}$ of 5 M NaOH for 4 days at 40°C and divided absorbance of the supernatant at 350 nm by the length of the *Daphnia* to yield absorbance/mm (adapted from ref. 35). Subtraction of an average measure of absorbance/mm obtained from three nonmelanic *Daphnia pulex* was used to account for absorbance due to compounds other than melanin. Comparison with a standard curve derived from commercial melanin (Sigma-Aldrich M8631) dissolved in 5 M NaOH was used to convert measures of absorbance to micrograms of melanin/mm *Daphnia*.

Gene Expression Assay. Relative expression of *Ddc* and *ebony* was assessed using real-time quantitative PCR. We identified all genes through BLAST searches on wFleaBase (<http://wfleabase.org>) with homologous *Drosophila* or crustacean sequence against the draft *D. pulex* genome sequence. Introns were identified by alignment with *D. pulex* cDNA sequence and, whenever possible, primers were designed to either amplify a region spanning at least one intron (to provide an additional method to check for amplification of contaminating DNA) or sit across exon junctions (to exclude contaminating DNA from amplification). Only primer pairs that yielded amplification efficiency greater than or equal to 97% were used for quantitation. Primer sequences are given in Table S4. For each clone-treatment combination, we isolated total mRNA from an average of 6 (range 1–12) individual *Daphnia*. We obtained expression data from four reference genes, the glyceraldehyde-3-phosphate dehydrogenase gene (G3PD), the α -tubulin gene, the RNA polymerase II gene (RNAP II), and the elongation factor 1-alpha gene (EF-1 α). Using the program geNorm, based on the methodology described by ref. 36, the three reference genes G3PD, α -tubulin, and RNAP II were determined to be the most stably expressed across all samples and were geometrically averaged to calculate a gene expression normalization factor for each sample.

We extracted total mRNA using an RNeasy Plus Mini kit (Qiagen) and confirmed the absence of DNA contamination by attempted PCR amplification of the RNA extraction in parallel with a positive control. Synthesis of cDNA was accomplished from 200 ng total RNA using the iScript cDNA Synthesis kit (Bio Rad), followed by quantitative real-time PCR performed in duplicate on $3\ \mu\text{L}$ of a 1:10 dilution of the cDNA synthesis reaction. We used a PCR mix with the

following final concentrations: $0.2\times$ SYBR Green dilution ($10,000\times$ SYBR Green I from Invitrogen, diluted to $5\times$ in DMSO), $1\times$ PCR Buffer, 0.2 mM each dNTP, $0.4\ \mu\text{M}$ forward primer, $0.4\ \mu\text{M}$ reverse primer, 2 mM MgCl_2 , and 0.04 U Immolase DNA Polymerase (BioLine). Thermal cycling conditions were 95°F for 2 min 30 s, followed by 40 cycles of 95°F for 20 s, annealing for 20 s, 72°F for 30 s and a plate read, followed by a melting curve from 60 to 90°F . Optimal annealing temperatures were determined empirically and the melting curve was examined for each reaction to confirm absence of DNA contamination and amplification of the desired template.

Statistical Analysis. To control our experimentwide error rate, we first performed multiple analysis of variance (MANOVA), predicting all traits as a function of clone nested within population, population nested within fish history, fish history, UV treatment, and fish history by UV treatment interaction, with clone treated as a random effect. Following highly significant results for all factors (all $P \leq 0.006$; Table S5), we performed an ANOVA on each trait separately, using the same model. Because clutch failure is nonnormal in a non-UV environment, we repeated our MANOVA without clutch failure and obtained comparable results. Before each analysis used in this study, we used Levene's test to formally assess equality of variances among all combinations of population and UV treatment. In addition, we used an Anderson-Darling test to formally assess normality of standardized residuals resulting from each analysis. We transformed data when necessary to satisfy these assumptions, and indicate the type of transformation in the text. Both tests indicated that our data satisfy equality of variances and normality of residuals for each MANOVA, ANOVA, and GLM analysis that we present. All analyses were performed in MINITAB.

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