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Genetic regulators of a resource polyphenism interact to couple predatory morphology and behaviour

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Phenotypic plasticity often requires the coordinated response of multiple traits observed individually as morphological, physiological or behavioural. The integration, and hence functionality, of this response may be influenced by whether and how these component traits share a genetic basis. In the case of polyphenism, or discrete plasticity, at least part of the environmental response is categorical, offering a simple readout for determining whether and to what degree individual components of a plastic response can be decoupled. Here, we use the nematode *Pristionchus pacificus*, which has a resource polyphenism allowing it to be a facultative predator of other nematodes, to understand the genetic integration of polyphenism. The behavioural and morphological consequences of perturbations to the polyphenism's genetic regulatory network show that both predatory activity and ability are strongly influenced by morphology, different axes of morphological variation are associated with different aspects of predatory behaviour, and rearing environment can decouple predatory morphology from behaviour. Further, we found that interactions between some polyphenism-modifying genes synergistically affect predatory behaviour. Our results show that the component traits of an integrated polyphenic response can be decoupled and, in principle, selected upon individually, and they suggest that multiple routes to functionally comparable phenotypes are possible.

1. Introduction

Phenotypic plasticity, the ubiquitous ability to adjust phenotypes to environmental signals, often exists as a composite of morphological, physiological or behavioural responses, and integrates diverse axes of trait variation [1–5]. For example, song phenotypes in many bird species depend on the social or behavioural environment and require specific morphological features and physiological or energetic capacities; without all three, song production may fail [6]. It follows that the functionality of composite phenotypes depends on the extent to which the components, or functional modules [7], of the phenotype work together, and failure to coordinate the requisite components can reduce functionality and fitness. For instance, spadefoot toad (*Spea multiplicata*) tadpoles that exhibit composite phenotypes intermediate between two well-coordinated resource-use ecomorphs are poorer competitors and are generally selected against [8,9]. Over evolutionary time, selection is expected to favour trait combinations that produce a fit, coherent organism [10]. Therefore, the underlying genetic and developmental mechanisms uniting component traits are important for understanding how these traits, associated with the same composite phenotype, can influence each other's evolutionary trajectory [11–13].

One way in which the evolutionary responses of composite phenotypes could be integrated is through shared or pleiotropic control of each

component's development by the same genes [14–18]. Such developmental organization can increase the likelihood that the component traits are expressed in tandem because only a single genetic switch needs to be activated [19]. This coordination should then increase functional integration and improve performance as compared with the activation of each trait individually [5]. In this way, the extent to which phenotypic components share common genetic bases is expected to influence the degree to which those components can be decoupled and evolve semi-independently. Advances in understanding the regulation of composite traits have been made largely by genotype-phenotype association tests (e.g. [20–23]), with functional validation of shared causal genes being less common (but refer to [24–26]). Therefore, validation of shared control still offers a crucial test of how composite phenotypes develop and can give new insights into how these phenotypes arise and change.

Composite plastic responses channelled through polyphenism, an extreme form of plasticity resulting in discrete, alternative phenotypes, have provided a useful context for exploring the shared control of component traits [27–29]. In the case of resource polyphenism, alternative morphs are induced by different environments and are characterized by traits that enable differential niche and resource use, highlighting the necessity to integrate behavioural traits for recognizing and acquiring different resources, morphological traits to handle different resources, and physiological traits to digest and process different resources [30]. Although the genetic basis of resource polyphenisms has generally been studied in terms of their morphology [31,32], attention to behaviour or physiology in the same polyphenisms can reveal how the response functions as a coordinated organismal phenotype [33,34]. Thus, the challenge remains to understand how different components of a resource polyphenism interact in a developmental genetic context. This understanding is needed to know how resource polyphenism responds to selection as a holistic or, alternatively, a compartmentalized phenotype. Here, we meet this challenge by combining behavioural assays with fine-grained morphological characterization in a system where multiple molecular regulators of resource polyphenism have been identified.

In the nematode *Pristionchus pacificus*, resource polyphenism involves a binary switch between feeding morphologies that specialize on alternative diets. This plastic response is induced by the abundance of bacterial food, concentration of conspecific pheromones and other metabolic cues [35–37]. The result is a developmental decision between two irreversible forms at the adult stage: the stenostomatous (St) morph, which feeds solely on microbes, and the eurystomatous (Eu) morph, which can also prey upon other nematodes. This polyphenism offers a fitness trade-off, whereby the St morph has faster development to adulthood, while the Eu morph has higher fitness than the St morph when forced to survive on nematode prey [38]. Following epigenetic licensing of plasticity in response to environmental cues [39], the induction of alternative morphs is regulated by a series of enzymes comprising the N-acetyl-α-glucosaminidases NAG-1 and NAG-2, the arylsulfatase EUD-1 and the cytosolic sulfotransferase SEUD-1/SULT-1 (figure 1) [31,40–42]. Manipulations of these genes toggle the switch, such that mutants and over-expression lines are fixed for one of the two naturally occurring morphs. Downstream of these factors, the switch decision is carried out by at least two nuclear receptors (NHR-40 and NHR-1) and a duplicate subunit of the Mediator complex, MDT-15.1 [29,43,44]. Although mutations in these three genes abolish the polyphenism, the morphologies produced differ from the wild-type morphs and constitute a range of forms intermediate between them (table 1; figure 2). In addition to their effects on morphology, polyphenism regulators also influence metabolic processes, including fat storage [29,45]. Furthermore, NHR-1 was shown to influence both predation ability and social aggregation [33], supporting the integration of morphology with behaviour. Thus, an examination of behavioural effects of mutations in polyphenism control genes, both singly and in epistasis, can test what parts of the polyphenism network regulate predatory activity and ability. Here, we used a series of behavioural assays for predatory activity and ability in a suite of *P. pacificus* polyphenism mutants to test this integration.

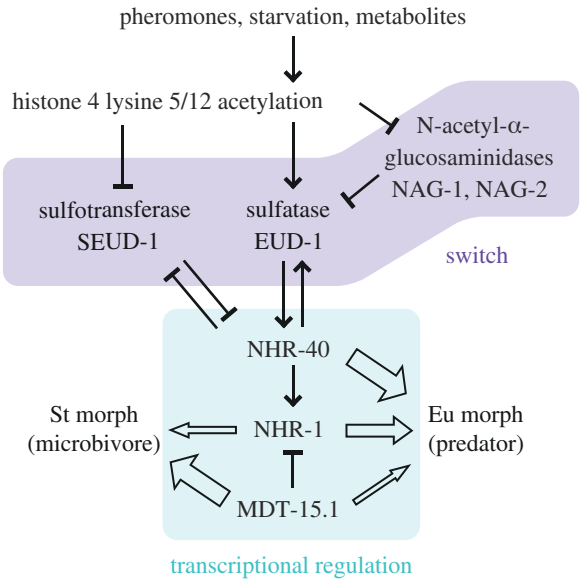
2. Methods

(a) Husbandry of predatory nematodes

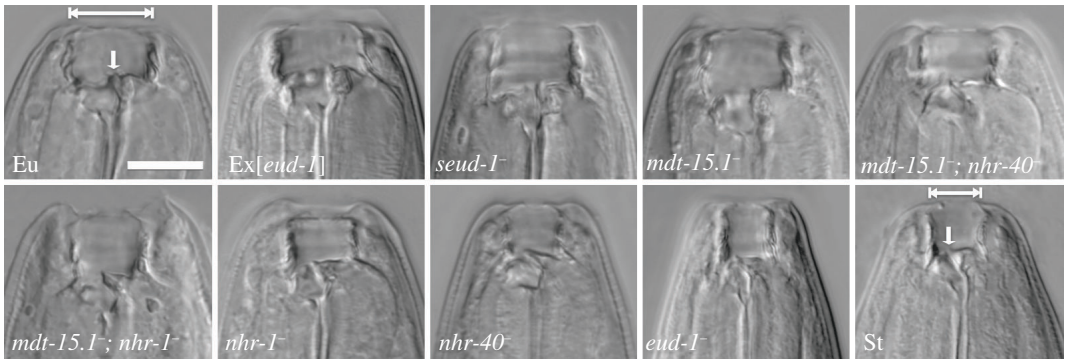
Individual *P. pacificus* nematodes for predation assays and morphometrics were reared on 6 cm nematode growth medium (NGM) agar plates each seeded with a bacterial lawn grown from 300 µl of *Escherichia coli* OP50 in L-broth. Culture plates were ensured to be free of bacterial or fungal contamination before assays and measurements. Nematode broods were allowed to grow until most individuals were adults, at which point there was still OP50 present, such that nematodes were allowed to continue feeding ad libitum and thus not be starved. Descriptions of the mutant strains assayed are given in table 1.

(b) Husbandry and preparation of prey nematodes

Caenorhabditis elegans N2 was used as prey for *P. pacificus*. Six NGM agar plates of *C. elegans* were allowed to grow past the point of starvation, resulting in a high density of young (primarily L1–L3) individuals. A 20 µm pluriStrainer (pluriSelect) was used to filter young larvae from eggs, older larvae and adults, after washing and pooling in M9 buffer from NGM plates. Filtered young larvae were collected into a 50 ml volume in a plastic tube that was left on a laboratory bench for approximately 1 h to allow nematodes to settle. The supernatant was removed until 1 ml remained, and the pellet of larvae was transferred into a microcentrifuge tube. The larval density was then quantified by pipetting 1 µl of the larva suspension onto a glass slide and counting the number of individuals using a compound microscope. This count was performed twice. The larvae were placed in a 6°C incubator to keep them healthy until use.



Regulatory model for polyphenism in *P. pacificus*. Following the switch-like activity of several enzymes (purple), two nuclear receptors and a Mediator subunit (blue) together affect one of two alternative adult morphs. The width of the arrows indicates the phenotypic contribution, as observed through mutants with intermediate morphologies, of each of the three latter factors to each of the two morphs. In addition to labelled relationships, *mdt-15.1* and *nhr-40* mutants interact to result in mouth morphologies not observed in either single mutant.



Mouth morphologies of the wild-type and constitutive mutants of *P. pacificus*. Image panels are ranked to include the Eu morph, as categorically assigned (Eu morph of strain PS312, Ex[*eud-1*], *seud-1* mutants), a grade of constitutive, intermediate forms (*mdt-15.1* mutants, *mdt-15.1; nhr-40* double mutants, *mdt-15.1; nhr-1* double mutants, *nhr-1* mutants), and the St morph, as categorically assigned (*nhr-40* mutants, *eud-1* mutants, the wild-type St morph of the RS5200B). Focal plane is sagittal; dorsal is right in all images. Double-sided arrows in first and last panels indicate different mouth widths in the Eu and St morph; the short arrow indicates the tip of dorsal tooth. In addition to mouth width and dorsal tooth shape, only the categorical Eu morph has a right subventral tooth (not fully visible in focal plane shown), another feature also captured by geometric morphometrics in this study. Scale bar, 10 µm.

Polyphenism regulatory network mutant phenotypes. Mutations in various genes influence production of alternative morphs in *P. pacificus*. See also figure 2.

eud-1(tu450); Ex[*eud-1(iubIs16)*]	Eu	[31,45]
seud-1(iub7)	Eu	[40]
mdt-15.1(iub19)	intermediate (Eu-like)	[29]
mdt-15.1(iub29); nhr-40(tu505)	intermediate	[29]
mdt-15.1(iub28); nhr-1(tu515)	intermediate (St-like)	[29]
nhr-1(tu515)	intermediate (St-like)	[44]
nhr-40(tu505)	St	[44]
eud-1(tu445)	St	[31]

(c) Behavioural event definitions

Predatory activity was characterized by three event categories: attempted bites, successful bites and feeding, following assays used previously to assess *P. pacificus* predatory behaviour [38,46,47]. An attempted bite was identified by increased pharyngeal movement, a lack of breakage of the cuticle and a restriction prey of movement. A successful bite was identified by increased

pharyngeal movement, latching motion and successful puncturing of the cuticle of the prey item. Finally, feeding was scored as the consumption of the entrails of a prey item whose cuticle had been broken. Although events were nested, such that feeding events were also successful bites, and successful bites were also attempted bites, events were scored only as the most exclusive category. For statistical analysis, all three types of events were grouped (i.e. as attempted bites) to increase power and together make up the predatory activity of an individual.

(d) Assay for predatory activity

Prey larvae were placed onto clean 6 cm NGM plates at a density of ~1000 larvae per assay plate. The larvae were then allowed to acclimate to the plate for 30 min prior to introduction of the *P. pacificus* individual to be assayed. Healthy adults were individually chosen from culture plates and moved from their culture plate to an unseeded NGM staging plate. In the case of wild-type (non-mutant) strains with mixed morphologies present on culture plates, mouth morphology of individuals was determined using a Zeiss Discovery v. 20 stereo microscope at 225× magnification [48] prior to relocation onto separate staging plates. After acclimation of prey larvae, the *P. pacificus* individual was moved to the assay plate and allowed to acclimate for 10 min. Following this acclimation period, the *P. pacificus* individual was observed under a Zeiss Discovery v. 20 at 225× magnification for 10 min, with predatory activity recorded as the number of each of the three types of behavioural events defined above. At the end of the 10 min observation period, the plate was set aside for the predatory ability assay.

(e) Assay for predatory ability

The predatory ability assay began at the end of the observational period for the predatory activity assay. Here, the *P. pacificus* individual remained on the same plate with larval prey for 3 h. After this period, the number of prey corpses was quantified using a Zeiss Discovery v. 20 microscope at 225× magnification. During this process, corpses were categorized as immobile larvae with a frayed or torn appearance, which has also been described as 'deflated' [46], thereby distinguishing killed larvae from the occasional, otherwise dead larvae. Killed nematodes on all plates were counted twice and the lower of the two counts was retained.

(f) Assay for environmental effects

To determine whether rearing conditions influenced predatory activity and ability, strains that have been genetically modified to canalize each of the two morphs were reared under three alternative sets of conditions that, in the wild-type, induce different morph ratios. These strains were (i) a *eud-1* loss-of-function mutant, which is constitutively St under all environmental conditions previously tested, and (ii) a line with an integrated transgene over-expressing *eud-1*, *eud-1*$^{-}$; Ex[*eud-1*], which is constitutively Eu. Six culture plates were started from multiple foundresses each, so that after one generation there were many gravid hermaphrodites from which to collect eggs. Eggs from these plates were harvested using standard NaOH/bleaching protocols [49]. Eggs were quantified from 2 µl of egg suspension pipetted onto a glass slide and examined under a compound microscope. Once quantified, ~1000 eggs were placed in 10 ml of M9 liquid culture, which induces a high bias towards the St morph compared with other laboratory-rearing conditions [37], and ~500 eggs were placed on an NGM plate spotted with 300 µl of OP50, which induces a high Eu-bias. To ensure adequate numbers of healthy adults for the predatory assays, we prepared 10 liquid culture vials and 3 NGM plates per strain. After 4 d, the NGM plates were assayed for predatory activity and ability as described above as behavioural events and corpse assays, respectively. Once cultures reached starvation conditions, as assessed by ~4–5 d without food and most adults having observably smaller body size than under well-fed conditions (between days 7 and 9 post-bleaching), individuals were assayed to assess the effect of starvation on predatory behaviour. Starvation prior to the adult stage, when the dimorphism is irreversibly expressed, induces a higher frequency of the Eu morph in wild-type strains [35,50], although its effects on constitutive mutants were previously unknown. Individuals reared in liquid culture were transferred to a 15 ml conical tube and centrifuged for 3 min at 1500 rpm 5 d post-bleaching. The supernatant was removed to leave 2 ml, and the remaining solution was pipetted across several empty NGM plates. These plates were treated as staging plates prior to predatory activity and ability assays described above.

(g) Geometric morphometrics

Geometric morphometric data consisting of raw landmark coordinates for several strains, when reared under standard laboratory conditions, were obtained from a previous study [29]: the Eu-biased, laboratory reference strain PS312 for the wild-type Eu morph, *mdt-15.1* mutants, *nhr-1* loss-of-function mutants, *nhr-40* loss-of-function mutants, *mdt-15.1; nhr-1* double mutants and *mdt-15.1; nhr-40* double mutants. Landmark data original to the present study, specifically of individuals of other strains reared under the same conditions as the above, were collected using previously produced images [50]: the St-biased isolate RS5200B for the wild-type St morph, and a line over-expressing *eud-1*, the integrated mutant rescue line *eud-1*$^{-}$; Ex[*eud-1*]. All samples used a previously defined set of 20 landmarks [29], and these data characterized the mouth shape, especially aspect ratio (width-to-height) and tooth shape, using both homologous and sliding landmarks. Data were obtained following a previously described protocol [51]. These data thus allowed the association of fine-grained variation in mouth morphology with variation in predatory activity and ability. Specifically, a principal component (PC) analysis was performed, following

alignment via generalized Procrustes analysis, to identify and characterize major axes of morphological variation, and then their relationship to predatory behaviour was tested (see §2(h) below). Original images and geometric morphometric data were collected for strains reared in different environmental conditions for our determination of how morphology versus environment *per se* affects predatory behaviour.

(h) Statistical analysis

Predatory behaviour assays were analysed using a generalized linear model, fitted with a Poisson distribution because they produced count data, in the base package of R v. 4.0.1 [52]. Specifically, for each analysis, a model was fitted in which the total number of bites (attempted + successful + feeding) or the number of corpses was the response variable and the predictor variable was morphotype, as categorically defined (Eu, St or intermediate), genotype (strain) or rearing conditions (liquid, standard or starved), according to the assay. The total number of bites corresponds to our assay of predatory activity and the number of corpses corresponds to our assay of predatory ability. Morph categories were assigned for switch-mutant genes (*eud-1*, *seud-1*) according to qualitative observations from previous studies [31,40], and morph categories for other mutants (*mdt-15.1*, *nhr-1*, *nhr-40* and combinations thereof) were assigned according to previous morphometric analyses [29,53]. This full model was compared with a null model that did not include the predictor variable, using a chi-square test through the 'ANOVA' function. If the full model was significantly better than the null model, a pairwise Wilcox test with false discovery rate correction for multiple testing was used to identify which groups, if more than two were evaluated, significantly differed from each other. In addition to these tests, the relationship between an individual's predatory activity and predatory ability using a similar analysis was also tested. Specifically, the number of corpses was used as the response variable and genotype and the total number of bites as predictor variables. As above, this full model was compared with a reduced model only including genotype.

The relationship between predatory behaviour and morphology was evaluated using a weighted Pearson correlation test with the 'cor.wt' function in the psych package. First, the mean and standard deviation of the number of bites and corpses per strain was calculated from the data above. Next, values were obtained for the first two principal components and centroid size for every geometric morphometrics sample, and the mean and standard deviation of PC1, PC2 and centroid size for each strain were subsequently determined. The correlation was weighted using standard errors.

To determine if morph-constitutive strains developed different morphologies when reared under different environmental conditions, a Procrustes ANOVA was performed to test for differences among groups in the R package geomorph [54], with 10 000 randomized permutations, and false discovery rate-corrected *p*-values to account for multiple testing.

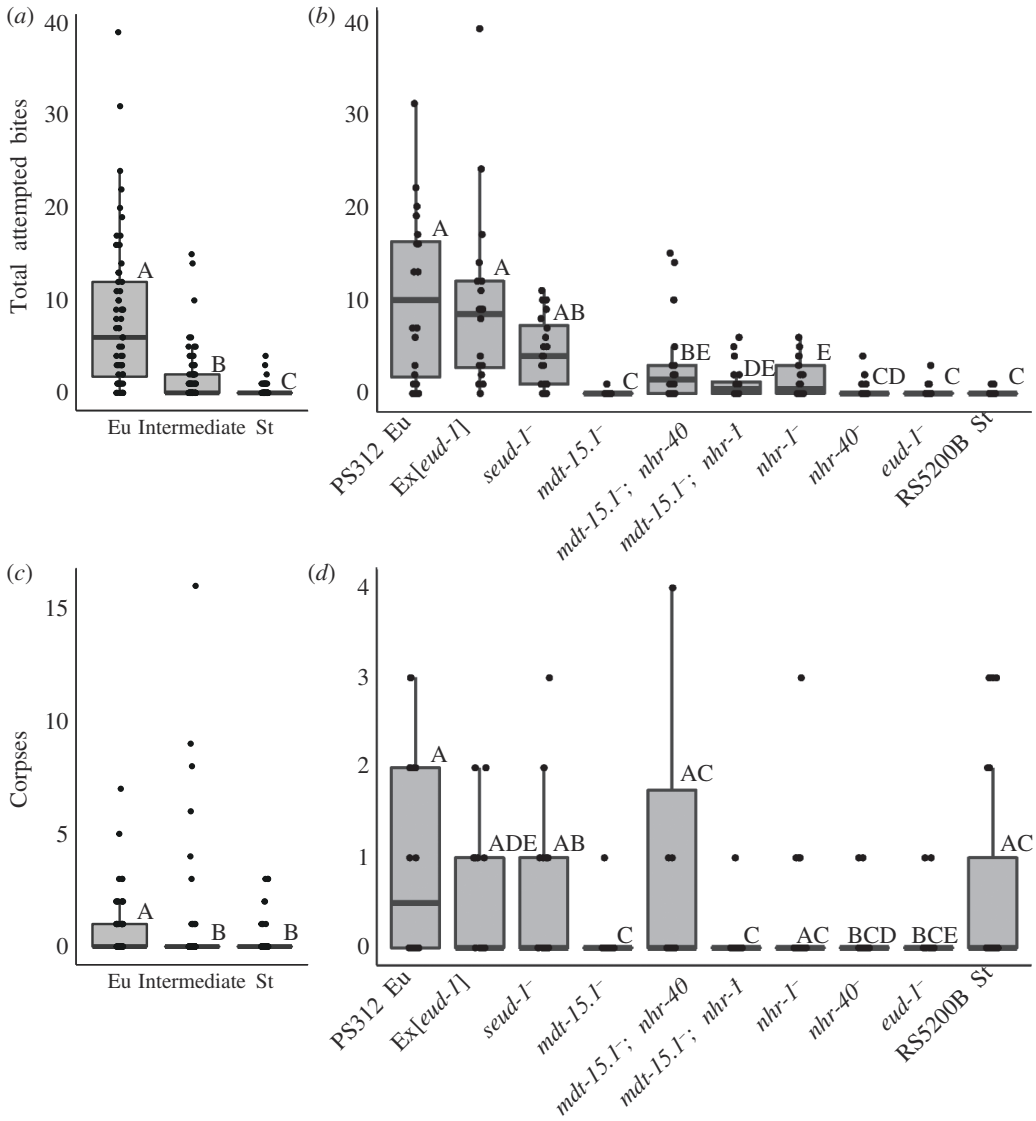
3. Results

(a) Morphotype predicts predatory behaviour

When we combined individuals based on previously described categorical morphology, we found that the Eu morph, whether in the wild-type or genetically manipulated strains, had significantly higher predatory activity, in terms of total bites, compared with St individuals ($p = 3.8 \times 10^{-15}$; figure 3*a*; electronic supplementary material, table S1). Mutants with intermediate morphologies, or those occupying an intermediate region of visualized morphospace, were intermediate in their predatory activity and significantly different from both the Eu morph ($p = 7.2 \times 10^{-11}$) and the St morph ($p = 0.00092$). When analysing the success of individuals over a longer period of time, as determined by the number of corpses after 3 h, Eu individuals produced significantly more corpses than St individuals ($p = 0.0019$) and individuals with intermediate morphologies ($p = 0.0019$; figure 3*b*; electronic supplementary material, table S2). However, the relationship between an individual's total bites and the number of corpses was not significant ($p = 0.9733$). In summary, individuals defined as Eu engage in the highest levels of predatory activity and show the highest predatory success, whereas those defined as morphological intermediates have some willingness to predate but are less effective at doing so. Thus, categorical morphotype, whether in wild-type individuals or fixed by various genetic manipulations, is a major predictor of predatory activity in *P. pacificus*.

(b) Polyphenism regulators differ in their influence on predatory behaviour

We next determined if strains with different morphological defects, owing to mutations in different parts of the polyphenism switch pathway, varied from each other in either predatory activity or ability. Most comparisons of individual strains were consistent with our finding above regarding Eu morph, St morph and intermediates, as categorically defined (electronic supplementary material, tables S3, S4). However, these comparisons also revealed differences within morphs, specifically in strains defined as intermediate in morphospace. Whereas *nhr-1* mutants, which show an St-like intermediate morphology [29,44], had predatory activity between that of canonical Eu and St morphs (figure 3*c*), *mdt-15.1* mutants, which are also intermediate and even closer to the Eu morph in form [29], showed activity levels that were not significantly different from the St morph ($p = 0.182$). Likewise, *mdt-15.1* individuals, despite having intermediate morphology, had minimal predatory success and were significantly different from wild-type Eu level ($p = 0.029$; figure 3*d*). Surprisingly, however, *mdt-15.1* mutants that also have the *nhr-1* mutation do exhibit predatory activity. Thus, *nhr-1* defects rescue predatory activity (*mdt-15.1* versus *mdt-15.1; nhr-1:* $p = 0.003$), although not ability, to the intermediate level of *nhr-1* single mutants (*nhr-1* versus *mdt-15.1; nhr-1:* $p = 0.720$), indicating epistasis of *nhr-1* over *mdt-15.1* in predation. Moreover, both predatory activity and ability are rescued in *mdt-15.1;*

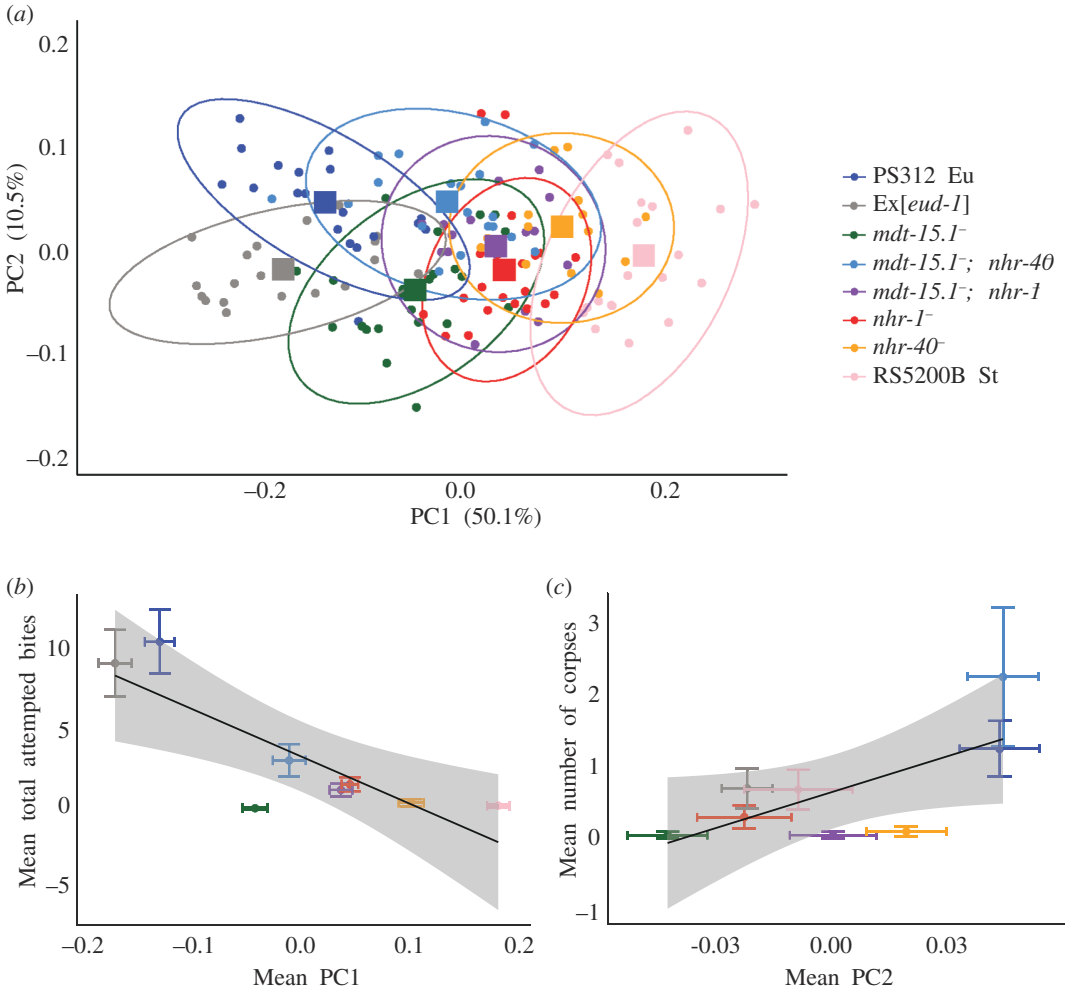


Predatory activity and ability by morph and strain. Shared letters denote groups that are not significantly different at a false discovery rate corrected alpha of 0.05. (*a*) Predatory activity by categorical morph. For the Eu morph, $n = 60$; for St, $n = 59$; for constitutive intermediates, $n = 80$. (*b*) Predatory ability by morph. Sample sizes are the same as in (*a*). (*c*) Predatory activity by strain. Sample size is $n = 20$ for all strains except RS5200B St, for which $n = 19$. (*d*) Predatory ability by strain. Sample sizes are the same as in (*c*).

nhr-40 double mutants, which display significantly more predatory activity than either single mutant (*mdt-15.1* versus *mdt-15.1; nhr-40*, $p = 0.0002$; *nhr-40* versus *mdt-15.1; nhr-40*, $p = 0.006$) and are statistically indistinguishable from wild-type Eu predatory ability ($p = 0.779$). Thus, individual polyphenism regulators, and the interactions among them, differently influence predatory behaviour.

(c) Two axes of morphological variation predict predatory behaviour

To determine whether fine-grained variation in morphology also predicts predatory behaviour, we characterized the mouth shape of eight genotypes using geometric morphometric data and associated them with genotype-specific measures of predatory activity and ability. We found that the first principal component of morphology (PC1) had high loadings from landmarks generally associated with mouth width (electronic supplementary material, table S5). Accordingly, genotypes were bounded along PC1 from those having wide mouths, with the wild-type Eu morph (strain PS312) and the *eud-1* over-expression line Ex[*eud-1*] on the one end, and those with narrow mouths, with the wild-type St morph (RS5200B) and *nhr-40* loss-of-function mutants on the other (figure 4*a*). Capturing another distinction between morphs, PC2 represented tooth position and shape (electronic supplementary material, table S5). When we tested the relationship of a genotype's mean position along PC1 or PC2 to predatory activity and ability (electronic supplementary material, table S6), we found that PC1 significantly correlated with the mean number of bites (Pearson's $r = -0.81$; $p = 0.014$; figure 4*b*), but not the mean number of corpses ($r = 0.36$; $p = 0.55$), indicating a higher willingness to predate but no increase in success. Furthermore, this correlation was specific to mouth width *per se*, as mouth size (measured as mean centroid size per genotype) neither correlated with its mean number of bites ($r = 0.37$; $p = 0.37$) nor its mean number of corpses ($r = -0.05$; $p = 0.91$). In contrast, PC2 did not show a significant relationship with bites ($r = -0.25$; $p = 0.39$) but had a nearly significant correlation with predatory ability ($r = 0.70$; $p = 0.053$; figure 4*c*). Thus, these two axes of morphological variation, mouth width and tooth shape, contribute to different aspects of predatory behaviour.



▨▨▨▨ ▨Effects of individual genetic regulators of polyphenism on predatory behaviour. (*a*) Fine-grained morphological variation among mutant strains. Plots combine previously collected and original data. Sample sizes: $n = 23, 17, 19, 22, 15, 20, 20$ and 19 for *mdt-15.1* mutants, *mdt-15.1; nhr-40* double mutants, *mdt-15.1; nhr-1* double mutants, *nhr-1* mutants, *nhr-40* mutants, Ex[*eud-1*], wild-type St morph of strain RS5200B, and the wild-type Eu morph of strain PS312, respectively. In general, PC1 is representative of mouth width and PC2 of tooth shape and position. A low PC1 shows a wide mouth, and a high PC1 shows a narrow mouth. A high PC2 displays nearby opposing teeth and a low PC2 has far non-opposing teeth. Dots denote individual samples, squares are the centroid for each genotype, and ellipses are the 95% confidence ellipse for each genotype. (*b*) Correlation of PC1 with predatory activity. A weighted Pearson correlation test showed a significant correlation ($r = -0.815$; $p = 0.014$). (*c*) Correlation of PC2 with predatory ability. A weighted Pearson correlation test showed a nearly significant correlation ($r = 0.701$; $p = 0.053$). For both (*b*) and (*c*), points represent mean values for each genotype, whiskers are the standard error of each genotype for each variable, and the black line is the smoothed linear fit with grey shading indicating standard error of the fit.

(d) Rearing environment alters predatory activity in morph-constitutive strains

Our final analysis investigated the effects of rearing environment on predatory activity and ability while controlling for genotype and morphology. Specifically, we used two strains that are constitutive for either the Eu or St morph and reared them under conditions that, in the wild-type, induce different morphs: a liquid culture medium that induces a high frequency of the St morph, and NGM agar culture, with either abundant food or dietary restriction, both of which induce the Eu morph [37]. Although rearing conditions had no effect on the number of corpses produced by a given strain (electronic supplementary material, table S7 and S8), there was a significant overall effect of rearing conditions on the number of total attempted bites for St-constitutive, *eud-1* mutants ($\chi^2 = 8.1093$; $p = 0.01735$). Although multiple comparisons with a false discovery rate correction failed to identify individually significant pairwise contrasts (figure 5*a*; electronic supplementary material, table S9), the only significant difference, when uncorrected, in the number of bites was between liquid and solid (i.e. well-fed) rearing media ($p = 0.039$), suggesting that this comparison drove the signal of overall significance in the model. Strikingly, the *eud-1* overexpression line showed St-like activity following development in a normally St-inducing environment, despite being constitutive for the Eu morph (figure 5*b*; electronic supplementary material, table S10). Together, these findings show that environmental conditions during development can cause a mismatch between behaviour and morphology in the *P. pacificus* resource polyphenism.

We also tested whether fine-grained morphological variation, beyond categorical morph (Eu or St) designation, could explain differences observed in predatory activity following different rearing conditions. Using geometric morphometric analysis, we found that the St-constitutive, *eud-1* mutant strain, when reared in liquid culture, had significantly different morphology than all other groups (figure 5*c*). Specifically, liquid culture exaggerated the narrow aspect ratio that characterizes the St morph. However, St morphology did not differ between the other two rearing conditions, which were standard (well-fed) or starved on solid-media culture plates (electronic supplementary material, table S11). Despite showing differences in predatory activity

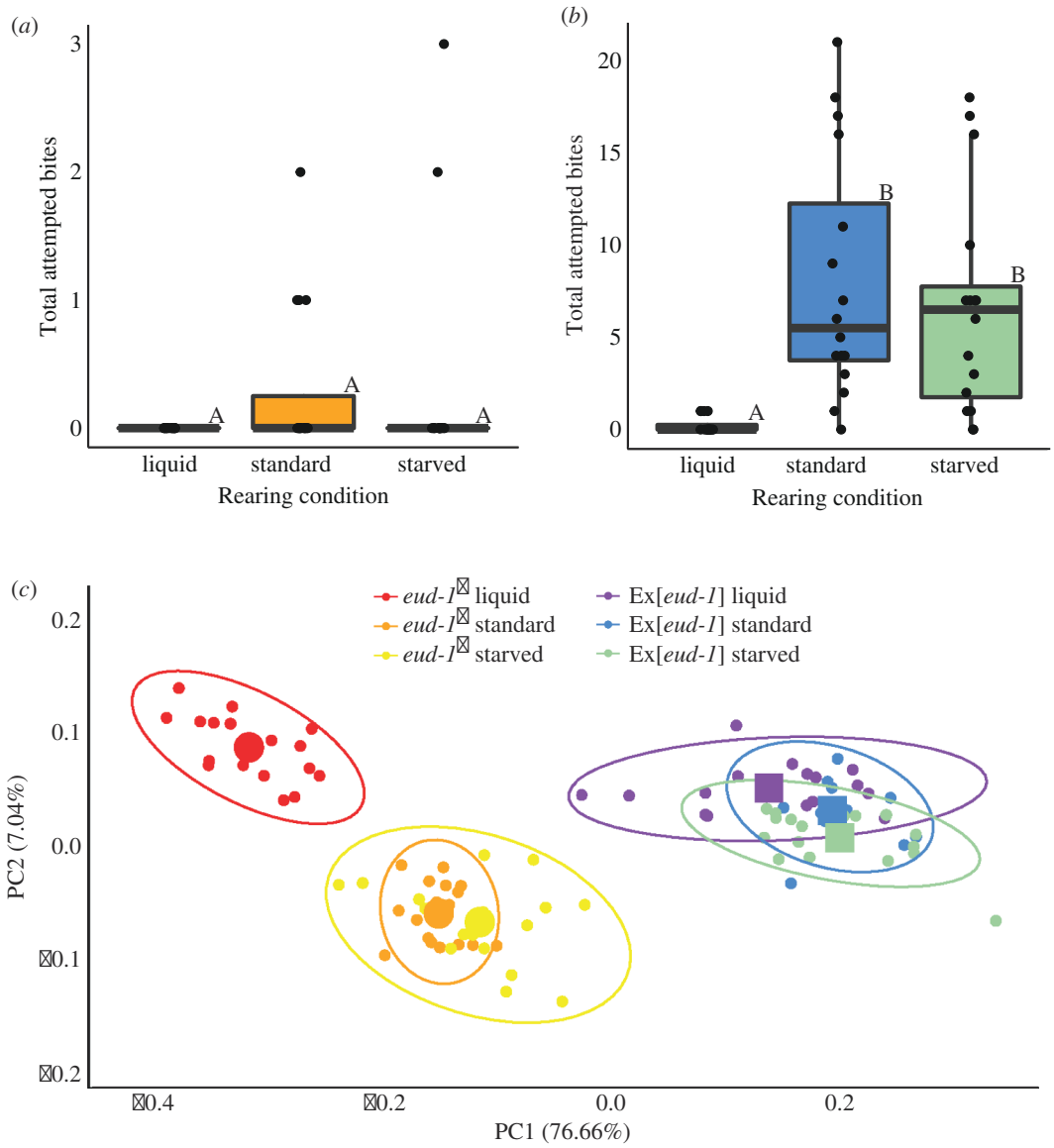


Figure 1. Effects of rearing environment on predatory activity in morph-constitutive strains. Rearing conditions were liquid culture, solid medium fed ad libitum (standard laboratory culture), and solid medium, starved. (*a*) Activity among rearing environments for St-constitutive, *eud-1* mutants. Sample size, $n = 16$ for each. Shared letters denote groups that are not significantly different at a false discovery rate corrected alpha of 0.05. (*b*) Activity among environmental conditions for the Eu-constitutive lines Ex[*eud-1*]. Sample sizes and annotations with shared letters as above. (*c*) Fine-grained morphological variation among different environmental rearing conditions for canalized mutants. PC1 separates St (low values) and Eu (high values). Dots denote individual samples, large circles are the centroid for *eud-1*$^{-}$ samples, large squares are the centroids for Ex[*eud-1*] samples and ellipses are the 95% confidence ellipse for each group. Sample sizes: $n = 18, 18, 17, 16, 15$ and 18 for *eud-1*$^{-}$ liquid, *eud-1*$^{-}$ standard, *eud-1*$^{-}$ starved, Ex[*eud-1*] liquid, Ex[*eud-1*] standard and Ex[*eud-1*] starved, respectively.

based on rearing conditions, the morphology of the constitutive Eu strain was indistinguishable across conditions. Therefore, morphology does not explain differences in predatory activity in Eu morphs raised under different conditions, showing that environment can decouple morphology from behaviour in this resource polyphenism.

4. Discussion

Through perturbations across a regulatory pathway for resource polyphenism, our study gives a molecular genetic context to the integration of a composite, plastic phenotype. To summarize, we found that: (i) categorically defined morphotype predicts the degree of predatory behaviour, which is graded between Eu, intermediates and St; (ii) perturbations of different parts of the polyphenism switch pathway differently influence predatory behaviour; (iii) two axes of fine-grained morphological variation predict different axes of variation in predatory behaviour; and (iv) interactions among morphology, genotype and environmental rearing conditions affect predatory behaviour. Our results thus show specifically how morphology, physiology and behaviour can be genetically decoupled, explaining how these components and resource polyphenism as a phenotype might together or independently evolve.

One key insight of our study is that independent axes of mouth form variation seem to differentially contribute to predatory activity and ability. The relationship between PC1, which is a proxy for mouth width, and the number of bites points to mouth width playing a role not only in the capacity of an individual to predate [38] but also being indicative of its willingness to do so.

One explanation for this might be pleiotropy of genes specifically controlling mouth width, such as factors acting downstream of the global polyphenism switch. Because there are different genetic routes to influencing mouth width independently of tooth morphology, it is possible that the network of genes affecting mouth width *per se* may also influence willingness to hunt. For instance, over-expression of *eud-1* simultaneously activates the switch in mouth morphology and foraging mode, which specifies the most extreme 'Eu'-like morphology of any mutant, as well as the highest levels of predatory activity. However, this higher activity is not reflected by higher ability. Instead, much of the variation in willingness to predate comes from mutants downstream of this switch, which have defects in their feeding morphology. This suggests that behaviour-influencing genes are activated both in parallel to and downstream of morphology-regulating genes.

An alternative explanation for the correlation of mouth width and predatory activity is that individuals have some ability to assess their own morphology and act accordingly. This possibility is supported by our observations that intermediate morphs, and the genotypes that produce them, differed from the canonical morphs in their willingness to bite. Analogously, caterpillars of the pipevine swallowtail (*Battus philenor*) change their body colour from black to red under high temperatures and, in turn, red caterpillars exhibit less refuge-seeking behaviour than black caterpillars [55]. This behavioural change is mediated through the effect of body colour on temperature, suggesting that the self-assessment of colour and concomitant altered behaviour is driven by different colours affecting perception of environmental information (i.e. temperature) for the behavioural change [56]. As illustrated by this example, two non-mutually exclusive mechanisms might explain how changes to one plastic trait can alter the development of another: cue-mediated change, whereby a change to one trait (e.g. of morphology) changes the state of the cue to which the second trait (e.g. of behaviour) responds, and response-mediated change, which occurs when a change in one trait changes the response of the second trait to a given cue [5]. While this idea awaits direct testing in *P. pacificus*, our results are consistent with response-mediated change such that mouth-width variation changes how predatory behaviour cues are perceived and processed. Such a test could involve assays of homozygous individuals of a given genotype with stochastic variation in their own morphology, which would determine the degree to which self-assessment occurs. In sum, our findings make testable predictions of what mechanisms guide the nematodes' decision to predate.

Our finding that tooth shape, but not mouth width, is related to predatory ability also suggests that different axes of morphological variation might have distinct roles in predation. Thus, to the extent that their genetic bases can be decoupled, they may be able to evolve independently. That alternative developmental modules can evolve in part independently in the context of polyphenism is well established [57–60], such that evolutionary change to one module need not require modification to others. Decoupling of feeding traits, in particular, can have important evolutionary consequences. For example, in damselfishes, decoupling of trophic traits leads to increased evolutionary rates of trophic morphology [61], and in diplogastrid nematodes, the loss of multiple ecomorphs is associated with increased rates of morphological evolution [62]. Moreover, specialization towards one feeding strategy may not necessarily come at a cost to another over evolutionary time, as suggested by an analysis of 18 North American bird species in which different aspects of morphology were related to complementary components of behavioural performance [63]. The relationships we observed in *P. pacificus* suggest that there is some degree of independent regulation of mouth width and tooth shape developmentally and that coordination of this regulation is important for successful feeding. We speculate that variation in the degree of coordinated regulation has contributed to the diversification of feeding morphologies within some *Pristionchus* species [64] and potentially even across Diplogastridae [62] that, in both cases, vary widely in combinations of mouth width and tooth structure. Testing this possibility will require behavioural assays like those performed here among lineages with greater morphological diversity, and ideally in combination with morphological manipulation via genetic perturbation.

In addition to the correlations of morphology individually to predatory activity and ability, these two measures may themselves be functionally linked, such as through associative learning. In *C. elegans*, multiple chemical and physical stimuli correlated with foods of different quality have shown that nematodes can learn by reward [65]. It is similarly possible that *P. pacificus* individuals that show high predatory activity when they are first introduced to prey fail to kill, reinforcing their unwillingness at repeated attempts, observed here as low ability after a 30 h exposure to prey. Indeed, the willingness of *P. pacificus* to bite *C. elegans* larvae can be dynamic, depending on the local presence of an alternative food source (bacteria) and the perception of competition [66]. Although we did not find a relationship between an individual's number of bites and corpse count, measurement of these variables through a time series may capture the flexibility of willingness to predate. The scaling of morphology to two measures of predatory behaviour, as we report here, thus allows tests of reward-based learning in this nematode system.

Another major insight of our study is that environmental conditions during development can cause a mismatch between morphology and behaviour. We found that an Eu-constitutive line exhibited the Eu morph across various environmental conditions that, in the wild-type, induce development of the St or Eu morph [37,50]. Strikingly, despite possessing Eu morphology, this strain exhibited St-like activity following development in St-inducing conditions. One explanation for this might be dietary conditioning, as the suite of solutes used to induce morph-bias in liquid and solid cultures differs [37]. Another reason might be social conditioning, as concentrations of pheromones are expected to differ between the two types of media, which support different population sizes per volume. Although all adults in our experiments were naïve to prey before being assayed for predation, pheromones signal competition with conspecifics [67] and potentially also the presence of potential prey nematodes, since some pheromone components are shared between *P. pacificus*, *C. elegans* and even more distantly related nematodes [36,68]. Not mutually exclusive with these explanations, the physical environment experienced by juveniles may affect adult behaviours. In *C. elegans*, locomotory transitions between crawling on solid media and swimming in liquid media are controlled by dopamine and serotonin, with serotonin promoting swimming and inhibiting crawling-specific behaviours [69]. Likewise, serotonin regulates decision-making and exploration of complex environments [70] and plays a crucial role in modulating feeding behaviour in *C. elegans* [71]. In *P. pacificus*, serotonin has not been implicated in the development of mouth

morphology but does mediate and coordinate pharyngeal pumping rate and tooth movement [46,47,71]. Thus, it is possible that serotonin-mediated changes for navigating a liquid culture environment might have pleiotropic effects on predatory behaviour. In this way, serotonin-based signalling might be a link between environmental sensing, especially liquid versus solid media, and regulation of predatory behaviour that operates independently from mouth morphology decisions.

The influence of environment apart from morphology suggests that the gene regulatory networks controlling predatory behaviour and morphology do not completely overlap. They also complement our suggestion above that the influence of mouth morphology on predatory activity, whether through genetic programming or morphological self-assessment, might be context-dependent and that environmental signals can override this influence. These observations indicate that the decoupling, and by extension, the capacity for semi-independent evolution, of component features of a composite trait can be affected by environmental conditions [72–75]. Such flexibility in integration is predicted to be favoured under the same conditions that favour the evolution of phenotypic plasticity—that is, heterogeneous environments [1]—and might act as another target for selective refinement of plasticity. More generally, these patterns of integration and environmental dependence of coordinated expression are expected to have important consequences for the ecology and evolution of plastic phenotypes [4,5]. Thus, our work supports the existing theory on the regulation of composite traits and provides a springboard for future investigations into the evolutionary causes and consequences of the patterns we observe.

Finally, our study supports the insight that a highly conserved coregulator of transcription in metabolic processes, *mdt-15/MED15*, interacts with nuclear receptors to influence behavioural traits. Not only has *mdt-15.1*, a duplicate homolog of *MED15* in *P. pacificus*, been identified as a key gene in the regulation of the polyphenism morphology, *MED15* is an essential regulator of metabolism in nematodes, including in response to nutrient-related stress [29,76,77]. Here, we found that *mdt-15.1* mutants exhibit a deficiency in their predatory activity compared with other mutants with intermediate morphology or the Eu morph. This result might be due to systemic issues of organism fitness, as hinted by the low fecundity of *P. pacificus mdt-15.1* mutants [29]. Yet, when the predatory activity of these individuals was compared with that of strains that had an additional mutation in either *nhr-1* or *nhr-40*, predatory activity was rescued. In the case of *mdt-15.1; nhr-1* double mutants, predatory activity was restored to the levels of *nhr-1* single mutant. In an extreme case of epistasis, *mdt-15.1; nhr-40* mutants showed Eu wild-type levels of predatory ability, even though the double mutants were intermediate in form and *nhr-40* single mutants are canalized for the St morph and show St-like predatory activity. This suggests that *mdt-15.1* and *nhr-40* mutations act synergistically to restore predatory behaviour despite that, individually, the same mutations result in St behaviour. Differences between *nhr-1* and *nhr-40* in their interactions with *mdt-15.1* are consistent with their only partially overlapping expression levels in the *Pristionchus* pharynx [44], which possibly mediate different parts of morphological and behavioural polyphenism. Furthermore, because *nhr-40* not only influences predation but also the willingness to aggregate through reduced aggression [33], defects in *mdt-15.1* may also interfere with behaviours more complex than biting alone. Given these three polyphenism regulators' overlapping but distinct effects on gene transcription and co-expression networks [29,45], transcriptomic comparisons including the two double-mutants can, in principle, pinpoint the observed epistasis in terms of downstream gene expression. Together, these results show that the relationships among polyphenism regulators are complex and that our ability to decouple morphology from behaviour suggests that multiple routes to the same functional outcome—in this case, predatory activity—are possible.

In conclusion, we have shown that decoupling of the plastic responses of a resource polyphenism is possible through the functional isolation of polyphenism's individual genetic regulators. Consequently, the possibility to separate normally integrated traits gives empirical footing to expectations about the genetic and functional integration of plasticity in specific and composite phenotypes more generally.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. The data and code supporting this article have been uploaded as part of the electronic supplementary material [78].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. R.M.N.: investigation, methodology, visualization, writing—original draft, writing—review and editing; N.A.L.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, supervision, writing—original draft, writing—review and editing; E.J.R.: conceptualization, funding acquisition, investigation, methodology, project administration, supervision, visualization, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

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