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Condition dependence of phenotypic integration and the evolvability of genitalic traits in a neriid fly

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The spectacular diversity of insect male genitalia, and their relative insensitivity to the environment, have long puzzled evolutionary biologists and taxonomists. We asked whether the unusual evolvability of male genitalia could be associated with low morphological integration of genitalic traits, by comparison with male somatic traits and female traits. We also asked whether this pattern was robust to variation in resource availability during development, which affects adult condition. To address these questions, we manipulated larval diet quality in a split-brood design and compared levels of integration of male and female genitalic and somatic traits in the neriid fly, *Telostylinus angusticollis*. We found that male genitalic traits were substantially less integrated than male somatic traits, and less integrated than female genitalic traits. Female genitalic traits were also less integrated than female somatic traits, but the difference was less pronounced than in males. However, integration of male genitalic traits was negatively condition-dependent, with high-condition males exhibiting lower trait integration than low-condition males. Finally, genitalic traits exhibited lower larval diet × family interactions than somatic traits. These results could help explain the unusually high evolvability of male genitalic traits in insects.

1. Introduction

A fundamental question in evolutionary biology is how complex structures evolve. Differences in morphological traits are typically low among closely related species, whereas male genitalia are often highly complex in form and function [1] and display a striking level of variation among species [2–7]. Explaining this diversity in insect genitalia has been a longstanding problem within evolutionary biology, and the selective pressures that shape these complex traits have been the subject of much debate [2,7–9]. The general consensus is that sexual selection is the main driver of genital divergence, as shown by comparative [10] and experimental [11] evidence. However, although sexual selection appears to drive genitalic divergence, the rapid evolution of male genitalia could also be facilitated by low morphological integration—a possibility that has received little attention.

'Evolvability', the potential for trait(s) to respond to novel selective pressures [12], is intimately related to modularity (i.e. the extent to which a trait or set of traits is able to vary independently of the rest of the phenotype). Modularity reflects the integration (i.e. degree of developmental covariation) of traits (see [13,14]). Morphological integration can be quantified by examining phenotypic or genetic correlations, and several studies have shown that integrated traits share a quantitative trait locus (QTL) [15–18]. While integration can be shaped by natural selection through the evolution of the genetic covariance matrix, the evolved degree and pattern of integration could affect subsequent evolvability.

We can expect functionally and developmentally related traits to evolve together through correlational selection [19]. Conversely, if traits are subject to contrasting patterns of selection (e.g. one trait is under directional selection and another is under stabilizing selection) then the dissociation of these traits in development is favoured, facilitating independent adaptation [20].

Insect genitalia may be expected to exhibit low morphological integration as a result of variation in the form of selection acting upon the different micro-structures that make up the genital apparatus [21]. This would generate high phenotypic variation in many genitalic traits [22]. Moreover, if past selection has acted to reduce genetic covariances among genitalic traits, then different components of the genitalic apparatus might be able to respond to selection relatively independently of other components. Integration in functionally independent traits has been regarded as a constraint, particularly for allometry, co-regulation of growth [23–27] and the evolvability of individual traits [28]. Research on integration, however, has been biased towards the study of mammals, whereas plants and arthropods are not well studied despite having very distinct modular body plans (for review, see Esteve-Altava [29]).

Using the neriid fly *Telostylinus angusticollis*, we manipulated the larval nutritional environment across 19 families to examine the patterns of integration of adult genitalic and somatic traits. We also asked whether differences in integration between trait types and sexes would remain consistent across both low- and high-nutrient larval diet treatments, which affect adult condition. Increasing the concentration of dietary nutrients during the larval stage results in increased body size and enlarged secondary sexual traits in *T. angusticollis* [30], but the condition dependence of genitalic traits has not been investigated before in this species. Based on the large differences in male genitalia among species in this genus [31], we predicted that male genitalic traits would be less developmentally integrated than other traits in males, and less integrated than female genitalia. Male genitalia appear to diversify more rapidly than female genitalia based on comparative morphology [10]. Moreover, in Diptera, *doublesex* (*dsx*), which is involved in genital disk development, exhibits a male-specific pattern of exon substitution that is consistent with 'runaway' evolution [32]. We also expected that rich-diet individuals (which possess more metabolic resources [33,34]) would exhibit greater developmental stability, resulting in higher trait integration. Weak environmental effects on genitalia could be another reason why genitalic traits tend to respond efficiently to selection. We therefore predicted that male genitalia would exhibit low effects of environment (larval diet) and weak family × larval diet interactions when compared to somatic traits and female genitalia.

2. Material and methods

Flies used in this experiment were derived from laboratory-reared stocks of *T. angusticollis* (Enderlein) (Diptera; Neriidae) that originate from individuals collected in 2017 at Fred Hollows Reserve, Randwick, NSW, Australia (33°54′44.04″S, 151°14′52.14″E) and were reared in the laboratory for four generations prior to this experiment. All individuals were reared in climate chambers at 25°C ± 2°C with a 12 : 12 photoperiod and provided with water every two days. Eggs from this population were randomly collected and reared using a nutrient-intermediate larval diet [30].

Virgin adults were collected at emergence, separated by sex and kept in 400 ml cages (maximum 30 individuals per cage) fitted with mesh stockings to allow for ventilation, a moist cocopeat substrate to provide humidity and access to sugar, yeast and water, *ad libitum*.

We used a full-sib, split-family breeding design. Randomly chosen virgin individuals were paired to create 17 mating pairs at 15 ± 2 days old. Each pair was allowed 48 h to mate within 120 ml cages provided with a nutrient-rich oviposition medium and access to sugar, yeast and water, *ad libitum*. Following the 48 h period, from each mating pair, we transferred 20 eggs to a nutrient-poor larval diet and 20 eggs to a nutrient-rich larval diet, also based on [33]. Upon emergence, virgin adult offspring were allowed 24 h for their exoskeletons to sclerotize fully and then frozen at −80°C for dissection and morphological measurements. We quantified 6 genitalic and 12 somatic traits on each of 93 males, and 4 genitalic and 11 somatic traits on each of 96 females [35]. All traits were quantified by measuring the lengths of the structures (see electronic supplementary material), except for testes (TE), for which we measured area in mm^2. For both sexes, we used thorax length as an index of body size [36]. To minimize the loss of samples for multivariate analyses, missing trait values (4.7% of total trait values in females and 5% of trait values in males) were replaced with the mean value for the family × larval diet × sex combination (where > three individuals were available for that treatment combination).

All analyses were carried out using R 3.5.3 [37]. For each set of traits (genitalic and somatic) and group combinations (sex and larval diet), we estimated morphological integration as the relative standard deviation of eigenvalues, $SD_{rel}(\lambda)$ [38]. The higher the value of $SD_{rel}(\lambda)$, the more variance is explained by the first few principal components of the trait matrix and therefore, the higher the integration. Integration was estimated from principal components analysis (PCA) performed separately on the correlation matrix for each sex × larval diet × trait type combination (electronic supplementary material, figures S3 and S4). Standard errors for integration values were obtained by a Jackknife ($n − 1$ traits) procedure. As a measure of environmental effects (i.e. larval diet quality), we computed marginal effect sizes, which indicate the variance explained by fixed effects [39]. To estimate the maximum genotype × environment interaction (G × E), we estimated the family × larval diet interaction. We computed conditional effect sizes, which reflect the variance explained by both fixed and random effects [39], and estimated the family × larval diet interaction by comparing the magnitude of the marginal and conditional effect sizes (see electronic supplementary material for details). Median parameter values were compared between treatment groups using nonparametric Kruskal–Wallis or Wilcoxon tests, with trait as the unit of replication.

3. Results

Morphological integration was higher in somatic traits than in genitalic traits for both males and females, although the difference was much greater in males (figure 1 and table 1). In males, both genitalic and somatic traits were more integrated in rich-diet individuals. Females exhibited a similar pattern, but the difference was significant only for somatic traits. Male genitalia were less integrated than female genitalia overall (Kruskal–Wallis test; $H = 4.8235$, d.f. = 1, $p = 0.028$), as well as within rich-diet (Kruskal–Wallis test; $H = 8.08$, d.f. = 1, $p ≤ 0.004$) and poor-diet treatments (Kruskal–Wallis test; $H = 8.08$, $p = 0.004$). Conversely, male somatic traits were more integrated than female somatic traits overall (Kruskal–Wallis test; $H = 15.652$, d.f. = 1, $p ≤ 0.0001$), as well as within rich-diet (Kruskal–Wallis test; $H = 15.652$, d.f. = 1, $p ≤ 0.0001$) and poor-diet treatments (Kruskal–Wallis test; $H = 15.652$, d.f. = 1, $p ≤ 0.0001$).
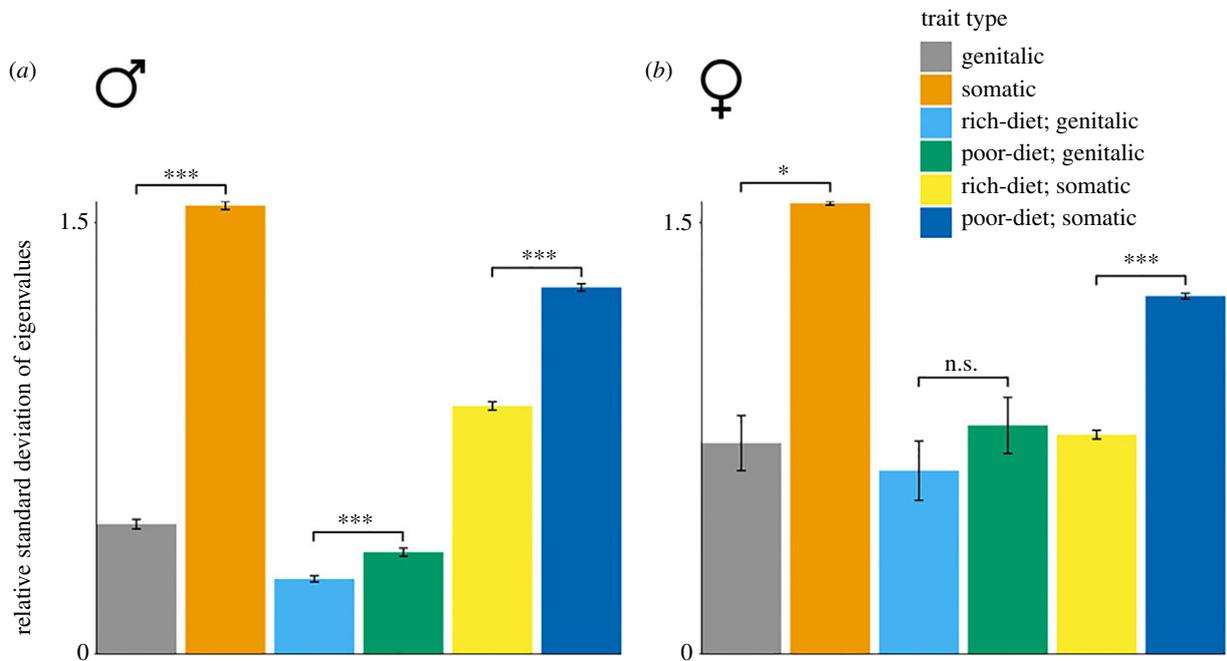
Figure 1. Trait integration, estimated as the mean relative standard deviation of eigenvalues, $SD_{rel}(\lambda)$, in genitalic and somatic traits of *T. angusticollis* males (*a*) and females (*b*) reared on nutrient-rich or nutrient-poor larval diets. Error bars represent standard deviation of the mean based on a Jackknife procedure. Significance codes are based on Kruskal–Wallis tests in electronic supplementary material, table S2 (0.0001, ***; 0.001, **; 0.01, *; >0.05, n.s.).

Table 1. Comparison of integration levels measured by the mean relative standard deviation of eigenvalues. The mean relative standard deviation is based on Jackknife resampling of PCA estimates with $n-1$, where $n =$ number of traits. M refers to the number of individual flies included in the sample. Kruskal–Wallis (KW)-tests compared Jackknife distributions of $SD_{rel}(\lambda)$ for genitalic versus somatic traits within each sex, or rich versus poor diet samples within each sex × trait type combination. Values in italics indicate significance ($p < 0.05$).

| dataset	*M*	*N*	mean SD$_{rel}$ (λ)	standard error of SD$_{rel}$ (λ)	Chi-squared *H*	*p*-value
overall differences in integration						
male; genitalic	93	6	0.461	0.044	*12.611*	*0.0004*
male; somatic	93	12	1.570	0.049		
female; genitalic	96	4	0.818	0.200	*9.375*	*0.022*
female; somatic	96	11	1.469	0.019		
condition dependence of integration						
male; rich-diet; genitalic	49	6	0.261	0.028	*9.016*	*0.002*
male; poor-diet; genitalic	44	6	0.354	0.038		
male; rich-diet; somatic	49	12	0.863	0.051	*17.28*	*<0.0001*
male; poor-diet; somatic	44	12	1.275	0.044		
female; rich-diet; genitalic	47	4	0.597	0.216	0.535	0.465
female; poor-diet; genitalic	49	4	0.745	0.204		
female; rich-diet; somatic	47	11	0.714	0.045	*14.286*	*<0.0001*
female; poor-diet; somatic	49	11	1.166	0.030		

We found no significant difference between males and females in the effect sizes of larval diet (marginal R^2) across all genitalic traits (Kruskal–Wallis; $H = 1.64$, d.f. = 1, $p = 0.201$), indicating that the magnitude of dietary effects is similar for male and female genitalia. However, effect sizes for larval diet were greater for male than female somatic traits (Kruskal–Wallis; $H = 4.25$, d.f. = 1, $p = 0.039$). Overall (sexes pooled), larval diet had a significantly larger effect on somatic than on genitalic traits (Kruskal–Wallis; $H = 16.12$, d.f. = 1, $p \leq 0.0001$) (figure 2). Comparison of marginal and conditional effect sizes suggested that larval diet × family interactions were relatively weak for both male and female genitalic traits, but substantial and significant for somatic traits (see electronic supplementary material for details).

4. Discussion

We found that male genitalic traits exhibited low integration by comparison with male somatic traits, as well as female genitalic traits. Conversely, male somatic traits were more integrated
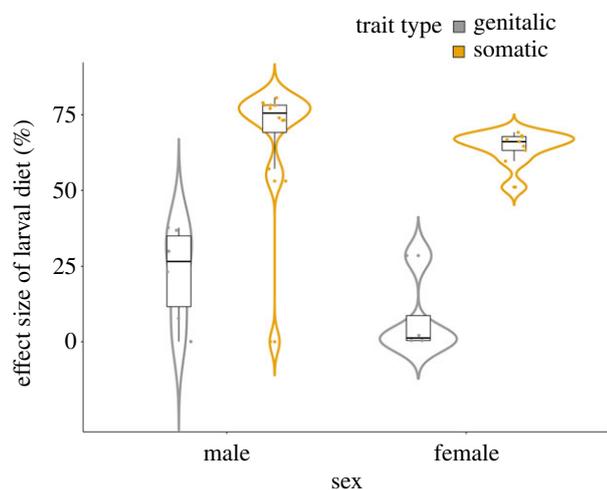
Figure 2. Marginal effect size of larval diet on genitalic and somatic traits in males and females. The violin plot outlines illustrate the kernel probability density (i.e. the width of the outlined area represents the proportion of the data located there). The box plots within the violin plots represent the median and interquartile range. Points represent average R^2 values for each trait.

than somatic traits of females. Both male and female genitalic traits were relatively weakly affected by larval diet and larval diet × family interactions, whereas somatic traits in both sexes exhibited substantial and significant larval diet × family interactions. This interaction is equivalent to the genotype × environment interaction (G × E) if among-family variance is entirely genetic and therefore estimates the maximum magnitude of G × E. Our findings thus suggest that environmental effects on the expression of genitalic traits are relatively weak and vary little across genotypes, by comparison with somatic traits. The low integration and weak environmental sensitivity exhibited by genitalic traits could help to explain the high evolvability of insect genitalia and the considerable differences in genitalic form often observed between closely related species.

Morphological integration [40] reflects the amount of variation that is available to selection and, thus, the independent evolvability of traits [12,41]. Low integration could therefore enhance the evolvability of male genitalic traits. Conversely, high integration could reduce maladaptive variability in somatic traits. If less integration does indeed equate to less constraint on evolution, we suggest that male genitalia evolve more rapidly than male somatic traits at least in part because of differences in evolvability rather than merely the strength of selection, as suggested previously by some authors (e.g. [42]). For example, genitalic trait shape evolves faster than somatic trait shape in *Onthopagus* beetles [43]. We suggest that this difference in evolutionary rates could be related to lower integration among genitalic traits and between the genitalia and body size, resulting in less functional constraint on genitalic evolution. Differences in the shape and relative size of micro-structures that comprise the genitalic apparatus could therefore drive speciation via changes in genitalic form [44].

Several studies suggest that patterns of integration can evolve in response to natural selection [45]. For example, there is evidence that natural selection has favoured reduced integration in mammalian skulls and numerous human characters, and that this allowed sets of traits to respond to separate selection pressures to a greater extent than would otherwise be possible [46–49]. Past natural selection might have favoured low genetic covariance between male genitalic traits because

high variability in the sizes of different genitalic components within individuals increased the probability of individuals successfully mating with a wide range of females that varied in body size or other trait(s), thus increasing individual male fitness [50]. Since male genital morphology influences male mating success in a number of insect taxa [9,51,52], and since male fitness is typically more limited by mating success than is female fitness, selection for compatibility could potentially explain low integration of male genitalic traits. Once evolved, this low integration could then enhance male genitalic evolvability and facilitate the diversification of these traits. Selection favouring increased genitalic complexity in polyandrous groups [53] might also have acted to reduce the integration of formerly highly correlated and relatively simple genitalic traits. There is some evidence that the insect genitalia evolved from the modification of a primitive appendage of a common ancestor to all arthropods [54]. Selection for compatibility with a range of female phenotypes might have promoted differentiation of various components of the genitalic apparatus, resulting in reduced genitalic integration as a by-product and, in turn, facilitating further diversification through sexual selection. Here, we show that genitalic traits exhibit lower phenotypic integration in *T. angusticollis*. Establishing the generality of this pattern across the insects will require studies on other species spanning the range of insect diversity.

Weak environmental effects could also be a reason why genitalic traits tend to respond efficiently to selection [55]. Canalization against the effects of environmental factors such as larval diet ('environmental canalization' [56]) would tend to expose standing genetic variation to selection, facilitating rapid evolution [57,58]. We found that the larval diet × family interaction had a much larger effect on somatic when compared to genitalic traits (electronic supplementary material, figure S5). Surprisingly, the larval diet × family interaction was also found to have a significant effect on somatic traits in females but not males, possibly indicating sex-differences in genetic architecture. While this illustrates the typical pattern of low condition dependence of genitalic traits, it is also possible that somatic and genitalic traits may compete for shared and limited resources during development (see electronic supplementary material for further discussion).

Overall, we found that low-condition (poor-diet) males had higher genital and somatic trait integration than did high-condition (rich-diet) males. In females, trait integration was similarly affected by larval diet, although the effect was not significant for genitalic traits. Nonetheless, despite these effects of larval diet on trait integration, the difference in integration between genitalic and somatic traits was maintained. The negative condition dependence of trait integration shows that, contrary to our predictions, high-condition individuals do not invest their extra resources into enhanced developmental stability and canalization. Rather, it appears that differential condition dependence of different traits results in a reduction in trait integration in high-condition individuals (figure 1).

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