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Evolution of Shape by Multiple Regulatory Changes to a Growth Gene

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Abstract

Genetic changes responsible for morphological differences between species are largely unknown. Such changes can involve modifications of growth that are relevant to understanding evolution, development and disease. We identified a gene that induces male-specific wing size and shape differences between *Nasonia* wasp species. Fine-scale mapping and *in situ* hybridization reveals that changes in at least three regions (two strictly in non-coding sequence) around the gene *unpaired-like* (*upd-like*) cause changes in spatial and temporal expression of *upd-like* in the developing wing and corresponding changes in wing width. *Upd-like* shows homology to the *Drosophila unpaired* gene, a well-studied signaling protein that regulates cell proliferation and differentiation. Our results indicate how multiple changes in the regulation of *upd-like* are involved in microevolution of morphological and sex-specific differences between species.

INTRODUCTION

The diversity of animal size and shape has fascinated scientists for centuries (1, 2). Changes in morphology, such as the beaks of birds, toes of lizards and hands of humans, underpin diversification and adaptation into different ecological niches (3, 4, 5). Yet the genes and genetic changes responsible for animal shape differences between species remain poorly understood (6, 7, 8). Genetic studies have revealed some genes and pathways required for growth and regulation of size and shape during development, many of which are also involved in human disease such as cancer and diabetes (9). These include signaling genes that regulate organ-specific patterning and growth (e.g., Wnt, Hippo, JAK/STAT), nutritional and hormonal regulators of body size (TOR and insulin signaling), and effector genes of cell growth and proliferation (cell cycle, apoptosis, protein synthesis) (9, 10). However, it is not yet clear whether evolutionary changes in these genes or others underlie size and shape differences between animal species (7).

The parasitoid jewel wasp *Nasonia* is an emerging as a model system for investigating the genetics of species differences in development and morphology. The *Nasonia* genus consists of four closely related species, each of which has evolved a distinct male wing size (8,11–13). The greater than twofold difference in male wing size between *N. vitripennis* and *N.*

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Materials and Methods

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giraulti (Fig. 1A) provides a tool for investigating the evolution of growth regulation. We isolated the two largest effect quantitative trait loci (QTL) for this male wing size difference (11, 14); *wing-size1* (*ws1*) (14) and *widerwing* (*wdw*) (11), which increase wing size through different mechanisms. The *giraulti* allele of *ws1* increases overall male wing size relative to *N. vitripennis*. The genetic change underlying *ws1* has been mapped to a 13.5kb noncoding interval containing the 5'-UTR of the sex-determining factor *doublesex* (15, 16) where changes in *doublesex* expression level in the developing wing are correlated with the wing size difference (8). *doublesex* is a major sex-determining factor in animals, found from humans to worms, which provides the downstream signal of somatic sex (15) and which also can have a role in growth regulation (8, 16).

Positional cloning and identification of *wdw*

Widerwing (*wdw*) alters male-specific wing shape as well as wing size, by changing cell numbers differentially across the width of the male wing, but with no effect on female wing size (11). The male wing size phenotype was initially mapped to a 1Mb region by introgressing chromosome segments from *N. giraulti* into *N. vitripennis*. Recombination mapping using generated recessive lethal mutations linked to the introgression (Fig. S1) (17) mapped *wdw* further to 115kb (Fig. 1). During this process, we broke the region into intervals differentially affecting male wing width, which we made into inbred lines for further analysis (18). We also created a 135kb introgression (*wdw*-135k line) of *N. giraulti* sequence in *N. vitripennis*, resulting in males with wide wings which are not significantly different in wing width from the original *wdw* introgression line (Fig. 1).

Only one gene, *NV21594*, occurs within the 115kb *wdw* region (Fig. 1) (18), suggesting that it could be the gene behind the wing size difference. However, it is also possible that genes adjacent to the 115kb region are responsible, as their expression patterns could be encoded in *cis*-regulatory elements within this region. We tested the expression of *NV21594* and adjacent genes *NV10313*, *NV21595* and *hmm1064384* in wings using RT-PCR. We detected expression of all but *NV10313* in male wings (18). We further investigated whether *NV21594* or adjacent genes have spatial expression patterns in developing wings using RNA *in situ* hybridization (17). Whereas *NV21595* and *hmm1064384* do not show a spatial pattern of expression (Fig. 1) (18), *NV21594* shows a clear spatial expression pattern that differs between *N. vitripennis* and the *N. giraulti* introgression. Specifically, it is expressed in the tip of the *N. vitripennis* prepupal male wing but is expressed much more broadly along the wing edge both in the *giraulti* allele of *wdw* in a *vitripennis* background (*wdw_gV*) and in *N. giraulti* male wings (Figs. 1, S3, S4) (18). Thus, the correlation between expression pattern and wing size suggest *NV21594* as the likely candidate for *wdw*.

Through searches to other arthropod genomes, we have identified genes with homology to *NV21594* in the holometabolous insect orders Hymenoptera, Coleoptera, and Diptera (Fig. S5) (18), which include the *Drosophila melanogaster* gene *unpaired* (*upd*). The *upd* candidates from these orders share five conserved peptide motifs, including an unusual short motif (WxNPGG) that is diagnostic for *upd* candidate protein genes in *Nasonia*, *Apis*, *Tribolium* and *D. melanogaster* (Table S1) (18). We therefore refer to *NV21594* as *unpaired-like* (*upd-like*). The *upd* candidates occur in clusters with two paralogous genes (*upd-like2* and *upd-like3*) in each of these taxa, as is also found in *D. melanogaster*. These proteins are also highly alpha helical (Fig. S5), typical of cytokines (21). *Upd* in *Drosophila* regulates cell proliferation as a ligand of the JAK-STAT pathway, and is thus a reasonable candidate for a wing growth gene. *Upd* is thought to play the same role in *Drosophila* as the mammalian cytokine *Interleukin-6* (*IL-6*), which is involved a number of human diseases (21).

The *wdw_g* introgression both increases wing width (by 25%) and expands the length of the *upd-like* expression domain along the wing edge (by 159%) relative to *N. vitripennis* (Fig. S3). It may be that *wdw_g* introgression could be causing the observed expansion in *upd-like* expression, but it is also possible that this expansion is simply a byproduct of the larger wing. We thus examined *upd-like* expression using the large winged *wsI_g* QTL, which increases wing width by 30% (8). Although *wsI_g* and *wdw_g* have wings similarly wider than *N. vitripennis*, *upd-like* expression in *wsI_g* is *vitripennis*-like (Fig. S4). Specifically, expression is absent from the posterior wing margin and significantly contracted relative to *wdw_g*, with a 54% smaller expression edge length (Fig. S3). These results suggest that the *wdw* region is a driver of both *upd-like* expression and male wing size. In females, the *wdw_g* introgression does not affect wing size (11), and *upd-like* is expressed in a broad curve in both introgression and *N. vitripennis* female wings (Fig. S6). This indicates that *upd-like* is not a male-specific gene, but rather that its expression is regulated in a male-specific fashion. The dependence of both male wing size and expression pattern on the *wdw* allele strongly indicates that *upd-like* is the gene that mediates the wing size difference.

Multiple noncoding intervals around *upd-like* contribute to the wing size difference

Several recombinants within the 115kb *wdw* region had intermediate wing sizes, indicating that it is likely that multiple functional sequence changes contribute to the full *wdw* phenotype. Experimental crosses between recombinant lines that differ in *giraulti* sequence within the 115kb region around *upd-like* (Fig. S1) identified three subregions within the 115kb *wdw* region that differentially contribute to wing size (Fig. 2). The middle subregion (*wdw*-B) contains both noncoding differences as well as a few coding changes in the first exon of *upd-like*. However, two of the three subregions, *wdw*-A and *wdw*-C, contain exclusively noncoding DNA. We created a *wdw*-A recombinant line (*wdw*-A-50k) that contains only 50kb of *giraulti* noncoding sequence spanning the *wdw*-A subregion. These *wdw*-A-50k males have significantly larger wings than *N. vitripennis* (Fig. 2), suggesting that noncoding changes in the region are sufficient to alter wing size regardless of whether the *upd-like* protein coding sequence comes from *N. giraulti* or *N. vitripennis*.

The three *wdw* subregions appear to each regulate distinct spatial parts of the wing width difference. We measured changes in width along the wing Anterior-Posterior (AP) axis in our recombinant lines (Figs. 3, 4, S7) and documented that the full *wdw_g* introgression increases wing width in each sector along the AP axis, with a particularly strong effect in the anterior-central sector (Fig. 3). The three subregions subdivide this pattern. *Wdw*-A only affects wing size in the anterior half of the wing, and appears to contribute primarily to the size of the anterior-central sector (Figs. 3, S7). In contrast, *wdw*-B and *wdw*-C have broader effects on wing width, and their specific effects appear to depend on the genotype at other subregions. Specifically, while the spatial effects of *wdw*-A are similar whether we compare recombinants from the left side or from the right side of the region, the spatial effects of *wdw*-B and *wdw*-C (significant differences (Fig. S7) across all sectors or modestly spatially restricted effects) depended on the direction of the comparison. This suggests either that the subregions interact epistatically to produce the full wing size difference or that there are additional modifier QTL, most likely located between *wdw*-B and C. For subsequent analysis of *wdw*-C, we focused on a recombinant line (LB3-125) that contains *giraulti* sequence only at *wdw*-C. This line has a fairly uniform effect, a $7.4 \pm 0.2 \mu\text{m} - 11.0 \pm 0.9 \mu\text{m}$ (7–15%) increase in each wing sector relative to *N. vitripennis* (Fig. 4).

Wdw-A causes an increase in wing width in the anterior half of the wing. It also causes an anterior-specific change in *upd-like* expression, which is broad and *giraulti*-like in the anterior of *wdw*-A (*wdw*-A-50k line) prepupal wings yet contracted and *vitripennis*-like in the posterior (Figs. 3, S3). We verified that this was a shift in the anterior expression pattern

and not a shift in the A-P compartment boundary by costaining with an antibody against the posterior compartment-specific protein *Engrailed* (Fig. S3).

In contrast to *wdw*-A's spatial effects, *wdw*-C (LB3-125 recombinant line) increases wing width across each A-P axis region (Fig. 4). To our surprise, *upd-like* expression in prepupal wings is *vitripennis*-like in this line (Fig. 4), despite the increase in adult wing size. An alternative mechanism that could produce a change in wing size is a temporal expansion of *upd-like* expression. Indeed, a broad *giraulti*-like expression pattern emerges in *wdw*-C pupal wings at a stage where expression in the wing tip is absent in *N. vitripennis* (Fig. 4) (18). Together, these observations suggest that *wdw*-C is a late-acting regulatory element with a broad effect.

We inferred the effects of *wdw*-B even though we did not genetically isolate it as cleanly as *wdw*-A and *wdw*-C. *Upd-like* has a broad expression pattern in recombinants bearing *giraulti* alleles at *wdw*-B+C (LB3-208 line), not significantly different from the full introgression (Figs. S3, S9). These data suggest that this subregion controls the early expression pattern while *wdw*-C controls the late expression pattern. This expression pattern also suggests that both *wdw*-A and *wdw*-B activate *upd-like* expression in anterior cells, perhaps in an overlapping pattern.

Interspecific patterns of *unpaired-like* expression and wing size

Male wing size and *upd-like* expression were compared in three *Nasonia* and two large-winged outgroup species, the closely related *Trichomalopsis sarcophagae* and more distant *Muscidifurax raptorellus* (Fig. 5). Small male wings appear to be derived, occurring in both *N. vitripennis* and *N. longicornis*, even though *N. longicornis* is more closely related to *N. giraulti* (12, 22). The reduced male wing width in *N. longicornis* appears to be due, in part, to evolution at the *wdw* locus: introgression of the *wdw_I* region into *N. vitripennis* (*wdw_IV*) increases adult wing size by 11%, intermediate between *N. vitripennis* and *wdw_gV* (11). Contraction of the *upd-like* expression pattern has occurred in both *N. vitripennis* and *N. longicornis* males (Fig. 5). Furthermore, the *N. longicornis upd-like* expression pattern change is also caused by evolution at *wdw*, as the *wdw_IV* introgression has a contracted expression pattern relative to *wdw_gV* (Figs. 5, S3).

Although the smaller wing size and *upd-like* expression in *N. vitripennis* and *N. longicornis* could be the result of a hybridization event between the species followed by introgression of the *wdw* region, the data do not support this. Sequence divergence in the three subregions is consistent with the species tree; there is 1.2% divergence between *N. longicornis* and *N. giraulti*, less than the respective 2.4% and 2.8%, between these species and *N. vitripennis* (18). Furthermore, analysis of phylogenetic tree topology with a sliding window across the *wdw* region, as well as examination of parsimony-informative sites, revealed no evidence of sequence introgression between *N. vitripennis* and *N. longicornis* (18). These results indicate that regulatory evolution of *upd-like* probably occurred in two separate *Nasonia* lineages, either by parallel reductions in *N. vitripennis* and *N. longicornis* or by reduction in an ancestral *Nasonia* lineage followed by expansion in the *N. giraulti* lineage. This observation plus the role of three subregions that we have identified between *N. vitripennis* and *N. giraulti* suggests that *unpaired-like* is a hotspot for wing size evolution in *Nasonia*.

DISCUSSION

This study determined the genetic basis of *wdw*, a major component of the male-specific wing size difference between *N. vitripennis* and *N. giraulti*. Our data indicate that *upd-like* causes the spatial changes in cell proliferation and growth within the wing and that *upd-like* is a hotspot for size and shape evolution in *Nasonia*. *Unpaired (upd)* in *D. melanogaster* is a

ligand for the JAK/STAT pathway (20). This pathway's role in cell proliferation makes it a plausible target of morphological evolution, though it is not yet known whether *upd-like* mediates its effects in *Nasonia* through JAK/STAT or other pathways. *Nasonia upd-like* appears to be particularly susceptible to wing growth-altering changes, indicating that the gene might have a specialized role in specifying organ size in the growth gene network. Considering that many size and shape differences between animals are due to differences in cell numbers (9), *upd-like* genes could be hotspots of size and shape evolution in other species as well.

Using a phenotype-based positional cloning approach, we have identified two different major QTL genes (*wdw* and *wsI*) that are responsible for sex specific differences in wing development between closely related *Nasonia* species. Based on what is known about fly wing development (19, 23, 24), the alternative candidate gene approach would not have predicted the role of either *upd* or *dsx* in *Nasonia* wing size. Nevertheless, both genes appear to be homologs of functionally conserved developmental regulatory genes, which is consistent with the hypothesis that core developmental genes tend to be involved in morphological evolution (25, 26). It has also been argued that non-coding *cis*-regulatory changes could play a central role in developmental differences between species (27, 28). Our findings support this view, and further implicate growth-regulating genes in organ-specific size and shape evolution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. *Wdw* maps to the *unpaired-like* locus and affects its expression
a: Male *N. vitripennis* and *N. giraulti* have diverged in wing size. b: Genes in the 200kb region around *wdw* in *N. vitripennis*, including a Maverick transposable element that is absent in *N. giraulti* and does not appear to affect wing size (18). c: Recombinant genotypes (left) and phenotypes (right) map the full *wdw* effect to a 115kb region. The wing width of haploid males of two genotypes of interest was measured using crosses (Fig. S1) (17) between the original *wdw* introgression (solid orange bar) and: (i) *N. vitripennis*, (ii) an introgression recombinant on the left, (iii) an introgression recombinant on the right, and (iv) an introgression recombinant on both sides (wdw-135k). White: *N. vitripennis* genotype. Orange: *N. giraulti* genotype. Gray: genotype between markers is unknown. Dark blue bars and *: Statistically significant difference in wing width between genotypes, corrected for multiple testing (Bonferroni). Light blue bars: no significant difference. DFDen: Denominator degrees of freedom from REML ANOVA. Error bars show standard error. d: RNA *in situ* hybridization reveals that the *wdw* introgression (wdw-135k line) has broader *upd-like* expression than *N. vitripennis*, consistent with the larger wing size. No expression pattern is seen for neighboring genes *NV21595* and *hmm1064384*. Arrowheads denote

boundaries of *upd-like* expression. Quantification of the size of the expression domain is in Fig. S3. Scale bars: 200um.

Figure 2. Multiple intervals within the *wdw* region affect wing size
The *wdw* phenotype splits into at least three subregions (light dotted lines), each with significant effects on wing size. Heavy dotted lines mark sequence differences between three pairs of adjacent recombinant lines which best define these subregions. Also shown are two recombinants (wdw-A-50k and LB3-125, also used in Figs. 3 and 4) where recombination has isolated the wdw-A and wdw-C subregions.

Figure 3. Anterior-specific effects of the wdw-A subregion

a: The original *wdw* introgression affects the size of sectors across the width of the adult wing, whereas the *wdw-A* subregion only affects the anterior-central sector. The width of four anterior-posterior (A-P) sectors was measured for the genotype pairs indicated on the left (17). i: original *wdw* introgression compared with *N. vitripennis*, ii: *wdw-A-50k* compared with *N. vitripennis*. Dark blue bars and *: Statistically significant difference in sector width between genotypes, corrected for multiple testing (Bonferroni). Error bars show standard error. b: Diagram of the adult wing sectors used in part a and Fig. 4a. c: Illustration of *Nasonia* wing imaginal disc, which develops in a similar orientation to the adult wing. d: Adult wings reveal anterior-specific expansion in *wdw-A*. Dashed line marks position of median vein (inferred A-P boundary). Dotted lines mark wing edges. e: *wdw-A* also regulates anterior-specific expression of *upd-like* in prepupae. Dashed line marks wing tip

(A-P boundary, Fig. S3). Arrowheads denote boundaries of *upd-like* expression. Scale bars: 200um.

Figure 4. Temporal regulation of *upd-like* by *wdw-C*

a: A recombinant line containing the *giraulti* allele of *wdw-C* (LB3-125) is wider than *N. vitripennis* in each of four sectors across the adult wing. b: Expression of *upd-like* before and after pupation reveals a temporal shift in *wdw-C*. In *N. vitripennis*, *upd-like* expression in the tip of the wing is visible only in gray larvae and prepupae. In pupal wings, expression is only apparent in the proximal posterior wing (arrow). In *wdw-135k*, expression is similar to *N. vitripennis* in gray larvae but expands apically in prepupae and is active later into pupation. In *wdw-C* (LB3-125), expression is *vitripennis*-like in the prepupa and *giraulti*-like in the pupa, suggesting that this subregion controls temporal regulation of *upd-like*. Control gene *wingless* is expressed at all stages (Fig. S8).

Figure 5. Evolution of wing size and *unpaired-like* expression

a: Adult wings and *upd-like* expression in prepupal wings from *Nasonia* males and outgroup species. Both *N. vitripennis* and *N. longicornis* males have a contracted expression pattern. The species cladogram is adapted from (29). b: A *N. longicornis* introgression of *upd-like* into *N. vitripennis* also shows a contracted expression pattern.