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Development of a wingless morph in the ladybird beetle, *Adalia bipunctata*

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Abstract

Many taxa of winged insects have independently lost the ability to fly and often possess reduced wings. Species exhibiting natural variation in wing morphology provide opportunities to investigate the genetics and developmental processes underlying the evolution of alternative wing morphs. Though many wing dimorphic species of beetles are known, the underlying mechanisms of variation are not well understood in this insect order. Here, we examine wing development of wildtype and natural wingless morphs of the two-spot ladybird beetle, *Adalia bipunctata*. We show that both pairs of wings are distally truncated in the wingless adults. A laboratory population of the wingless morph displays heritable variation in the degree of wing truncation, reflecting reduced growth of the larval wing discs. The coexistence of variable wingless morphs supports the idea that typical monomorphic wingless insects may be the result of a gradual evolution of wing loss. Gene expression patterns in wing discs suggest that the conserved gene network controlling wing development in wildtype *Adalia* is disrupted in the dorsoventral patterning pathway in the wingless morphs. Previous research on several species of ant has revealed that the anteroposterior wing patterning pathway is disrupted in wingless workers. Future investigations should confirm whether interruptions in both taxa are limited to the patterning pathways found thus far, or whether there are also shared interruption points. Nevertheless, our results highlight that diverse mechanisms of development are likely to underlie the evolution of wingless insects.

Keywords

Asynchronic development, histology, immunohistochemistry, morphometrics, wing patterning

Introduction

The evolution of flight is an important innovation which has contributed to the success of insects (Wagner and Liebherr 1992). Nevertheless, secondary loss of flight ability has occurred independently and repeatedly in most winged insect orders (Roff 1990) and is often accompanied by loss of wing tissue. In some cases variability in wing morphs is found within species, with examples of both genetic polymorphisms and environmentally induced polyphenisms (e.g. Braendle et al. 2006). Such species provide opportunities to investigate the genetics and the developmental processes underlying the evolution of alternative wing morphs. To date, ants and aphids have been studied in this light. Abouheif and Wray (2002) found that the conserved gene network controlling wing development in winged reproductives of various species of ants was disrupted at different points in the anteroposterior (AP) wing patterning pathway both across and within species to produce wingless worker castes. A genome-wide study of gene expression in nymphs of the pea aphid reported that hundreds of genes, mostly related to energy production, were differentially expressed between winged and wingless morphs (Brisson et al. 2007). Interestingly, about 75% of these genes were only differentially expressed in either polyphenic females or in genetically polymorphic males. In other words, one in four genes showed differential expression in both sexes. Thus, the polyphenism and the polymorphism share some, but not all, gene products with respect to the development of the wingless morph.

The order of beetles (Coleoptera) is a group of insects providing opportunities to extend this knowledge. Flight has been lost in more than thousand species (Roff 1994a) of which some exhibit wing dimorphism (e.g. Darlington 1936; Smith 1964; Dybas 1978; Hammond 1985). Although studies on darkling beetles, including the model species *Tribolium castaneum*, have contributed to understanding the development of beetle wings (Hundertmark 1935; Quennedey and Quennedey 1990, 1999; Tomoyasu et al. 2005), the developmental regulation of alternative wing morphs in this group remains largely unexplored. The two-spot ladybird beetle, *Adalia bipunctata*, is suited to address this issue, because wingless phenotypes occur occasionally in the wild (Majerus and Kearns 1989; Marples et al. 1993). Both the elytra (fore wings) and hind wings are reduced, but other body parts show no apparent morphological changes (Fig. 1B-D, bottom). Earlier work found that this trait is under the genetic control of a recessive allele at a single major locus (Marples et al. 1993). We have collected two beetles with this phenotype, and an individual heterozygote for the trait, from a single locality in three different years. It is highly unlikely that the recessive alleles they carried each represented a different recurrent mutation in the wingless gene, rather a single recessive allele for winglessness appears to be maintained at a low frequency in at least some wild populations. Its study could then help in understanding the evolution of winglessness or wing dimorphism in beetles.

Laboratory populations of the wingless morph in *A. bipunctata* show continuous variation in wing reduction, ranging from individuals exhibiting complete winglessness through to others which lack only small pieces of wing tissue. Nevertheless, we will consistently use "wingless" to describe this genotype. The variation in the degree of winglessness is affected by the genetic background (Ueno et al. 2004) and by environmental factors (Lommen et al. 2005).

In this paper, we examine the developmental processes underlying genetic wing dimorphism in *A. bipunctata* and genetic variation of wing reduction within the wingless morph. Wings can be reduced in a variety of ways, such as miniaturization, deletion of different parts of the wing, or a combination of both. Since the nature of reduction could provide candidate developmental mechanisms, we have first used comparative morphological studies to characterize the size and shape of the reduced wings in wingless adults. Because beetle wings begin to develop in the larva, we have then examined this process in wildtype and wingless phenotypes histologically. Finally, we have chosen three wing development genes known from *Tribolium* and other insects, and examined their expression patterns in both phenotypes.

Materials and methods

Insects

A laboratory stock of wingless two-spot ladybird beetles (*A. bipunctata*) was established by outcrossing two individuals (one a wingless homozygote and the other a heterozygote) found in Utrecht, The Netherlands, with more than a hundred wildtype beetles from the same population. We also started a pure wildtype laboratory stock from the Utrecht location (only for histological examinations of wildtypes, we used a laboratory stock originating from Koppert B.V.). All beetles were maintained in a climate cabinet at a constant temperature of 20.5ºC ± 1.5ºC (RH 65% ± 5%) and a photoperiod of 16L:8D. Both larvae and adults were fed with *Ephestia kuehniella* Zeller eggs with adults receiving a supplement of flower pollen.

Morphology of pupal and adult wings

We used wing colour patterns and wing structures as landmarks to compare wingless beetles with winged ones. To obtain landmarks on the elytra we crossed a winged mutant with a spotty colour pattern spanning the entire elytra (Majerus and Kearns 1989) (Fig. 1E) into the wingless stock. In this way, we reared over a 100 spotty wingless adults. Veins, hairs, sensory organs and pigmentation patterns served as landmarks in hind wings. To examine the size of reduced wings, we took morphometric measurements from 99 wingless adults (37 males, 62 females) and compared them to 73 wildtypes (35 males, 38 females). The wingless adults were chosen such that the range

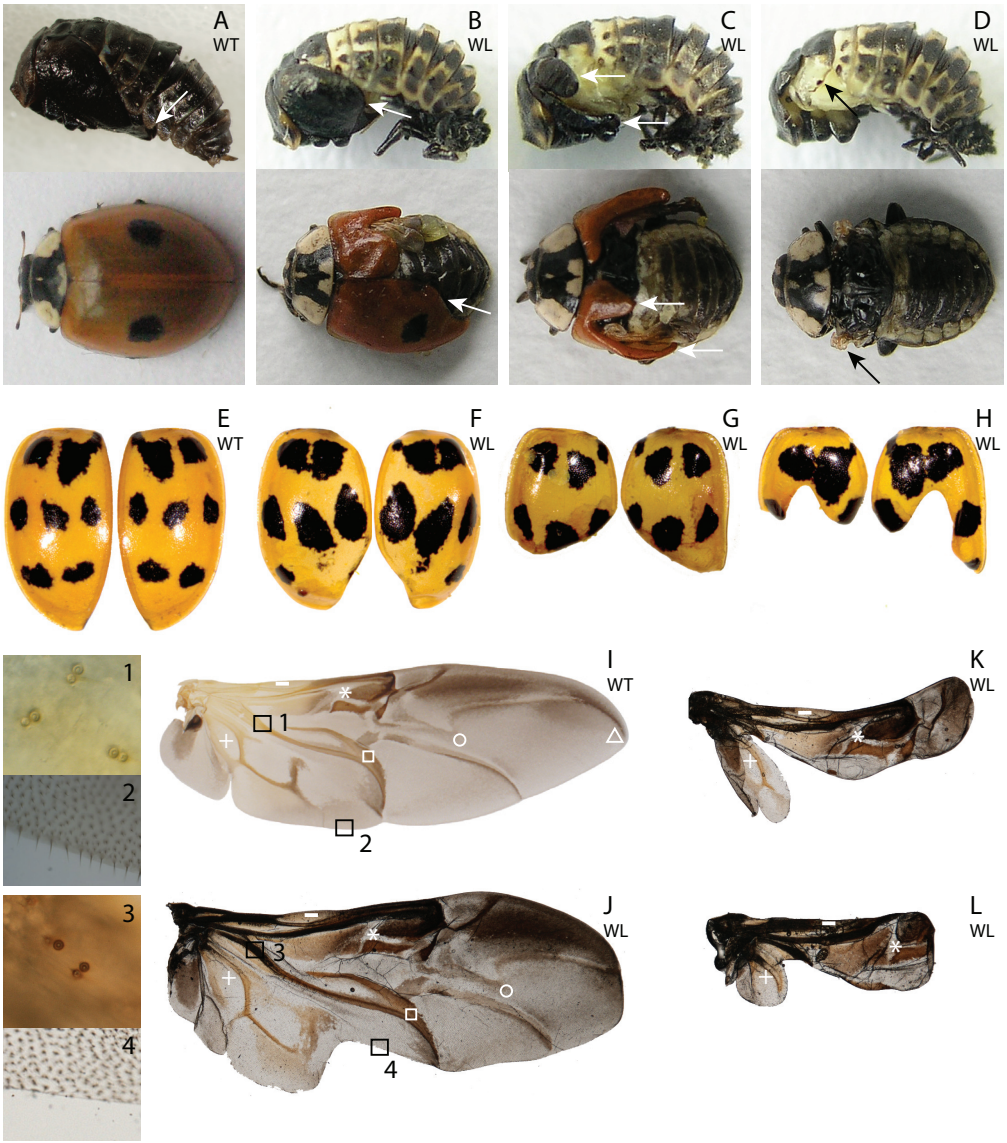


Figure 1. Wings of wildtype (WT) and wingless (WL) beetles demonstrate wing variation in *Adalia bipunctata*. A. WT pupa (top) and adult (bottom). Black drop-like structure in pupa is the wing case, with arrow indicating the distal tip. **B-D.** The size and shape of the pupal wing cases of WL beetles (white arrows in top pictures) correspond to that of elytra in the adults emerging from it (white arrows in bottom pictures). Pupa and adult in D. have no elytra tissue, but hind wings are visible (black arrows). **E-H.** Elytra of individuals homozygote for the *spotty* colour pattern allele indicate that elytra of WL beetles (F-H) are truncated rather than miniaturized, compared to beetles with wildtype wing development (E). **I-L.** The venation pattern and the pigmentation pattern on hind wings of WL beetles show that hind wings are also truncated rather than miniaturized. **I.** WT hind wing with 1. sensory organs on the Medial vein, and 2. long hairs at the posterior wing margin. Symbols represent some of the landmarks, either venation or pigmentation patterns. **J-L.** Hind wings of WL beetles. 3. shows that sensory organs on the Medial vein are similar as in WT. 4. shows that the posterior margin is truncated, since the black hairs typical of that margin in WT are absent. **B, C, H, and J-L** show wings with a typical "lobed" shape, with the wing consisting of two lobes.

of variation was captured in the sample. All insects were killed by freezing at –80^{0}C. On the wildtype adults the maximum length of the left elytron, and for wingless beetles the maximum length of the medial and outer part of the left elytron, were measured (Fig. 2A). Following that, the left hind wing was detached and mounted on a slide, and its maximum length was measured.
To better understand possible changes in the wing at the pupal-adult moult, we visually inspected the pupal wing cases (the cuticle covering the pupal wings, Fig. 1A) of 35 wingless and 35 wildtype beetles and compared them to the shape of the elytra in the adults after they had eclosed.

Histological examination of wing disc growth

We histologically examined the epidermis of the meso- and metathorax which contain the primordia (or wing discs) of elytra and hind wings, respectively (Fig. 3A), in larvae of wingless and wildtype phenotypes. Since we were also interested in variation within the wingless phenotype, we established three pure wingless families from parents lacking the major part of wing and elytron tissue. We used a scale from 0 (complete lack of elytra) to V (elytra approaching wildtype phenotype) to quantify their degree of winglessness as described by Lommen et al. (2005). All parents were scored as class 0, I or II (Table 1). About 60 larvae per family were used for histological studies and the remaining siblings were reared through to adulthood, and then scored for degree of winglessness. The same number of larvae from the Koppert wildtype stock was used for histological study.
Adalia bipunctata has four larval instars and pilot studies with wildtypes showed that wing disc growth does not begin until the third larval instar (L3). Therefore, we used larvae in stage L3, early-L4 (within 1 day after moulting), mid-L4 (1-2 days after moulting), and late-L4 (3-5 days after moulting, a few individuals were already a prepupa). Each wingless family and the wildtype stock contributed 7-29 larvae to every larval stage used. Larvae were anaesthetized with chloroform, and meso- and metathoraces were then fixed in 4%-paraformaldehyde in phosphate buffer (1x PBS, pH=7.2) for 2h. After dehydration in ethanol and Histo-Clear (Agar Scientific), the samples were embedded in paraffin, cut into transverse sections of 8-9 µm (Fig. 3A), stained with Mayer's haematoxylin and eosin, and photographed.

Gene expression patterns in wing discs

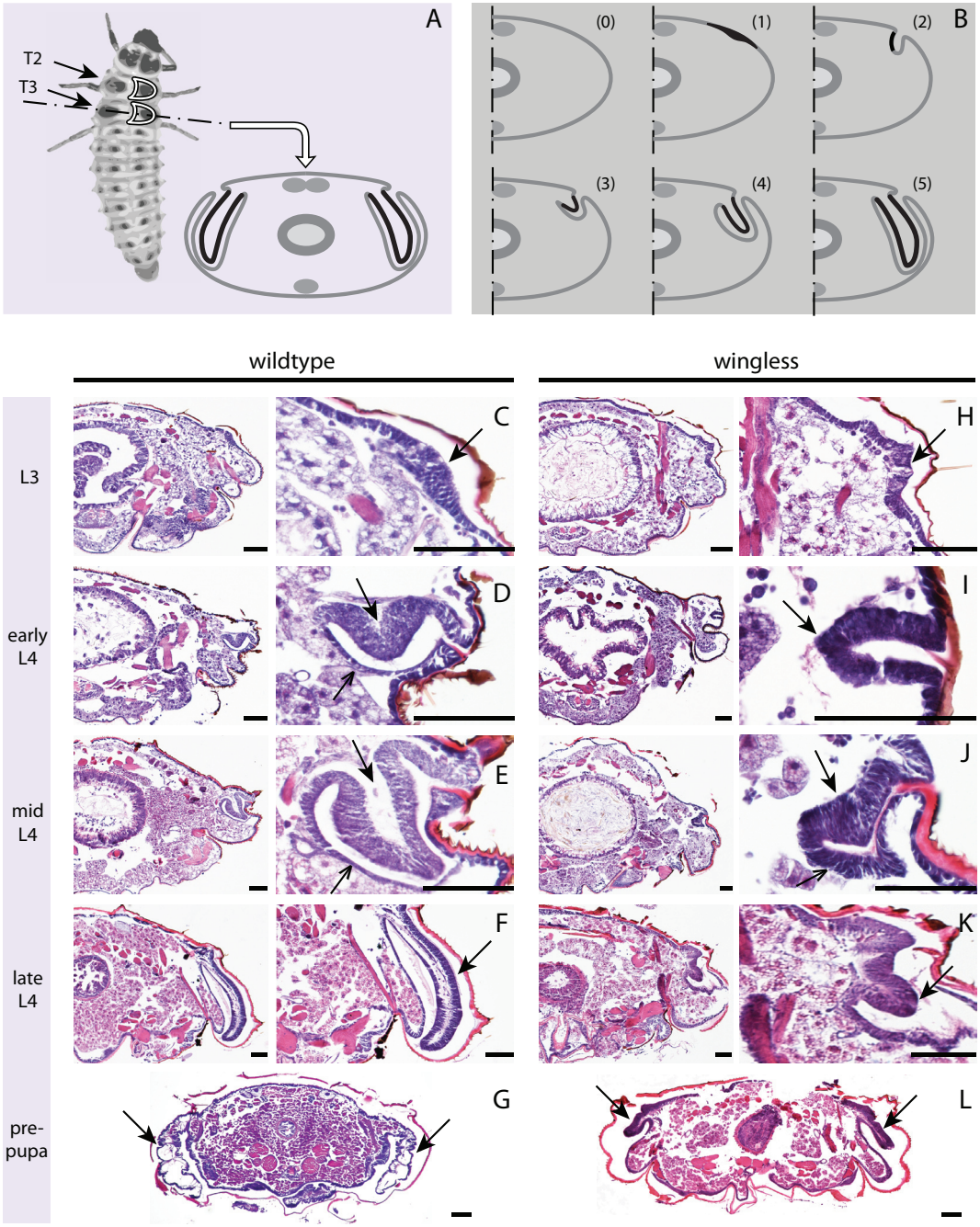
We investigated the expression patterns of *Distal-less* (*Dll*), *engrailed/invected* (*en*), and *pdm/nubbin* (*nub*) in larval wing discs, using antibodies against their protein products. We selected larvae from mid-L4 and late-L4 stages from the wildtype and wingless stock, since younger larvae yield insufficient tissue for dissection. They were anaesthetized with chloroform for 15 minutes and the thorax was then fixed for 30 min in 0.1 M PIPES (pH 6.9), 1 mM EGTA, 1% Triton x-100, 2 mM $MgSO_4$ and 9% formaldehyde. After excision of parts of the epidermis containing a wing disc,

the protocol of Brunetti et al. (2001) was followed for antibody staining. Samples of elytra and hind wings were kept separately in order to compare their gene expression patterns, and samples from the left and right side received antibodies against different gene products. We used a polyclonal antibody against Dll (1:100) (Panganiban et al. 1995), the monoclonal antibody 4F11 against En/Inv (1:100/1:200) (Patel et al. 1989), and a monoclonal antibody against Pdm/Nub (1:10/1:20) (Damen et al. 2002), and used Alexa Fluor 488 (1:200) and Texas Red (1:200) (Molecular Probes) as secondary antibodies. The samples were fixed for 30 min in 9% formaldehyde in wash buffer (cf. Brunetti) and were washed before the wing discs were separated from the epidermis and mounted in pure glycerol. Sample sizes were 5-20 for each combination of phenotype, wing type, developmental stage, and gene product. Exceptions were mid-L4 larvae from wingless beetles, from which we obtained only a few samples due to their minute size, and Nub, which was not tested in any mid-L4 larva. We also included unstained controls to check autofluorescence, and controls that only received secondary antibodies to test for non-specific binding. Images were obtained by confocal laser-scanning microscopy.

Statistical analysis

Measurements on adult wing sizes were analysed using the R statistical package (version 2.6.1., R Development Core Team 2007). Since the data of wingless beetles were not normally distributed and variances were unequal among groups, we applied non-parametric statistics or generalised models. We used a Spearman Rank Correlation test to reveal the relationship between maximum elytron and hind wing length for each sex in each wing phenotype. To compare this relationship between the wing phenotypes we then fitted regression lines to the data, using a Linear Model with Generalized Least Squares, fitted by Restricted Maximum Likelihood, to account for unequal variances. Maximum hind wing length was used as the response variable, while maximum elytron length, wing phenotype and sex were used as explanatory variables. This maximum model was then simplified by stepwise removal of non-significant factors, as revealed by Chi-square tests on the deviances, calculated by the log likelihoods, of the original and the simplified models. We used a Wilcoxon Signed Rank test to examine differences between lengths of the medial and outer parts of elytra of wingless beetles.

Histological data were analysed in SPSS 15.0. Using the histological transverse sections of wildtype larvae, we identified a set of successive morphological changes characterising wing development (see results and Fig. 3B). We then used this set to quantify the variation in wing disc growth of wingless beetles by scoring the development of the largest wing disc in each pair of wings in the transverse sections of each specimen. The largest elytron and hind wing disc differed in score in only 17% of the individuals, and not in any consistent pattern. Therefore, we only used the highest score of each specimen as an ordinal numeric variable for non-parametric analysis of wing disc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growth. For each larval stage dissected, we first tested by a Kruskal-Wallis test whether the three wingless families could be pooled. This was possible for stages L3 and early-L4, and the pooled data were then compared to the wildtype group by a Mann-Whitney U test. Because families could not be pooled for stages mid-L4 and late-L4, we used a Kruskal-Wallis test to examine overall differences among the three wingless families and the wildtype group, followed by multiple pair wise Mann-Whitney U tests with a Bonferroni correction. The same procedure was followed to test for differences among the three wingless families in the frequency distribution of the degree of winglessness of siblings reared to adulthood.

Results

Morphology of pupal and adult wings

Though size and shape of wings of wingless *A. bipunctata* are extremely variable, there are clear patterns in the mode of reduction. Both in the elytra and in the hind wings, morphological landmarks show that the wings are reduced by distal truncation, and in addition, a posterior part of the wings, variable in size and shape, is missing (elytra in Fig. 1F-H, compared to wildtype in E; hind wings J-L, compared to wildtype in I). Rarely, the anterior part of the wing is not well developed. Apart from deformations in size and shape, and sometimes in structure, the truncated wing of wingless beetles resembles the corresponding part in the wildtype wing. Thus, pigmentation patterns, veins, sensory organs, and hairs are present on truncated hind wings at the correct position (elytra in Fig. 1F-H, compared to wildtype in E; hind wings J-L, compared to wildtype in I).

Figure 3. Development of wing discs in larvae of wildtype (WT) and wingless (WL) *A. bipunctata*. **A.** The second (T2) and third thoracic segment (T3) of the larvae bear pigmented spots (black arrows), below which the wing discs develop (white outlines at the larva's right lateral). Larvae were cut transversely (dashed line) to obtain sections (cartoon at the right). The outline symbolises the epidermis, the inner circle symbolises the larval digestive track, and the wing discs are laterally in black. **B.** Cartoons showing subsequent characteristic phases of wing development, numbered 1-5. Drawings represent the right side of transverse cross sections through the larval meso- or metathorax. Wing disc growth starts with thickening of the epidermis (phase 1), followed by a first (phase 2) and second (phase 3) invagination that forms the wing which subsequently expands ventrally (phase 4) until it is more than 4x its width (phase 5). **C-L.** Transverse cross sections of wing discs in WT (overviews of half the body in left panels of **C-F**, with close-ups in the right panels, complete section in **G**) and WL phenotypes (overviews of half the body in left panels of **H-K**, with close-ups in the right panels, complete section in **L**) from larval stage L3 to the prepupal stage. Closed arrows point to the wings, open arrows to the wing sacs and bars correspond to 0.1 mm. **C, H.** In L3 epidermal thickening takes place in both phenotypes (phase 1). **D.** After the first invagination of the tissue has taken place (phase 2), a second invagination forms the wing disc and the remaining invaginated cells form the wing sac (phase 3) in early-L4 in WT. **I.** In early-L4, the WL morph has only undergone the first invagination (phase 2). **E.** The wing discs of mid-L4 WT larvae have increased in length (phase 4). **J.** Wing discs of mid-L4 WL larvae have just formed (phase 3). **F.** Late in L4, the wing sac has broken and the wing disc of WT larvae has expanded further (phase 5). **K.** In WL morphs late in L4, the size of wing disc is smaller than that of WT. **G.** In WT prepupae, the wing disc has folded and covers the lateral side of the body. **L.** In WL prepupae, the wing discs have a typical reduced shape and left and right side differ in size.

The length of the truncated elytra and hind wings is highly correlated (r_s=0.700 for males; r_s=0.908 for females; both p<0.001) (Fig 2B). However, comparing the maximum lengths of elytra and hind wings in wingless beetles to those of wildtypes, shows that hind wings are more reduced than elytra (Chi-square, p<0.001) (Fig. 2B). In addition, both the elytra and flight wings of many wingless beetles have a distinct "lobed" shape, consisting of two lobes (Fig. 1B, C, H, J-L, 2A). This has never been observed in adult wildtype beetles. In each sex, the outer lobe is typically larger than the medial lobe (males: median outer and medial lobe are 1.60mm and 0.70mm, respectively; females: 1.88 mm and 0.97 mm; Wilcoxon Signed Rank test, both p<0.001) (Fig. 2C).

The size of the wing cases was reduced to a variable extent in pupae of wingless beetles (Fig. 1B-D) compared to those of winged ones (Fig. 1A). The degree of reduction displayed in each pupa and the shape of the case matched that of elytra in the eclosing wingless adult (Fig. 1B-D, compare top with bottom), suggesting that wing reduction took place before pupation, that is, in the larval stage.

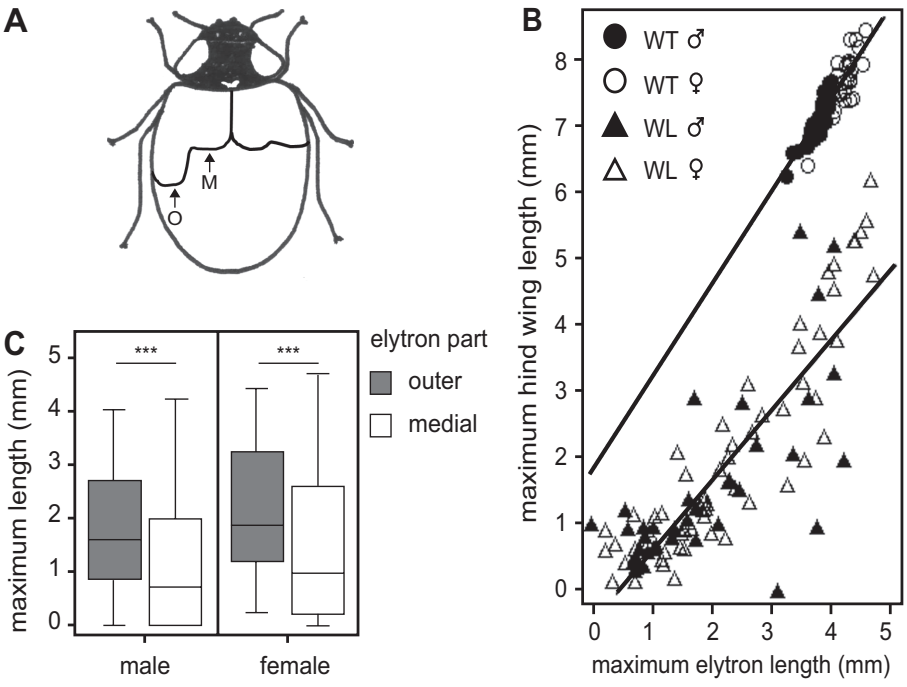


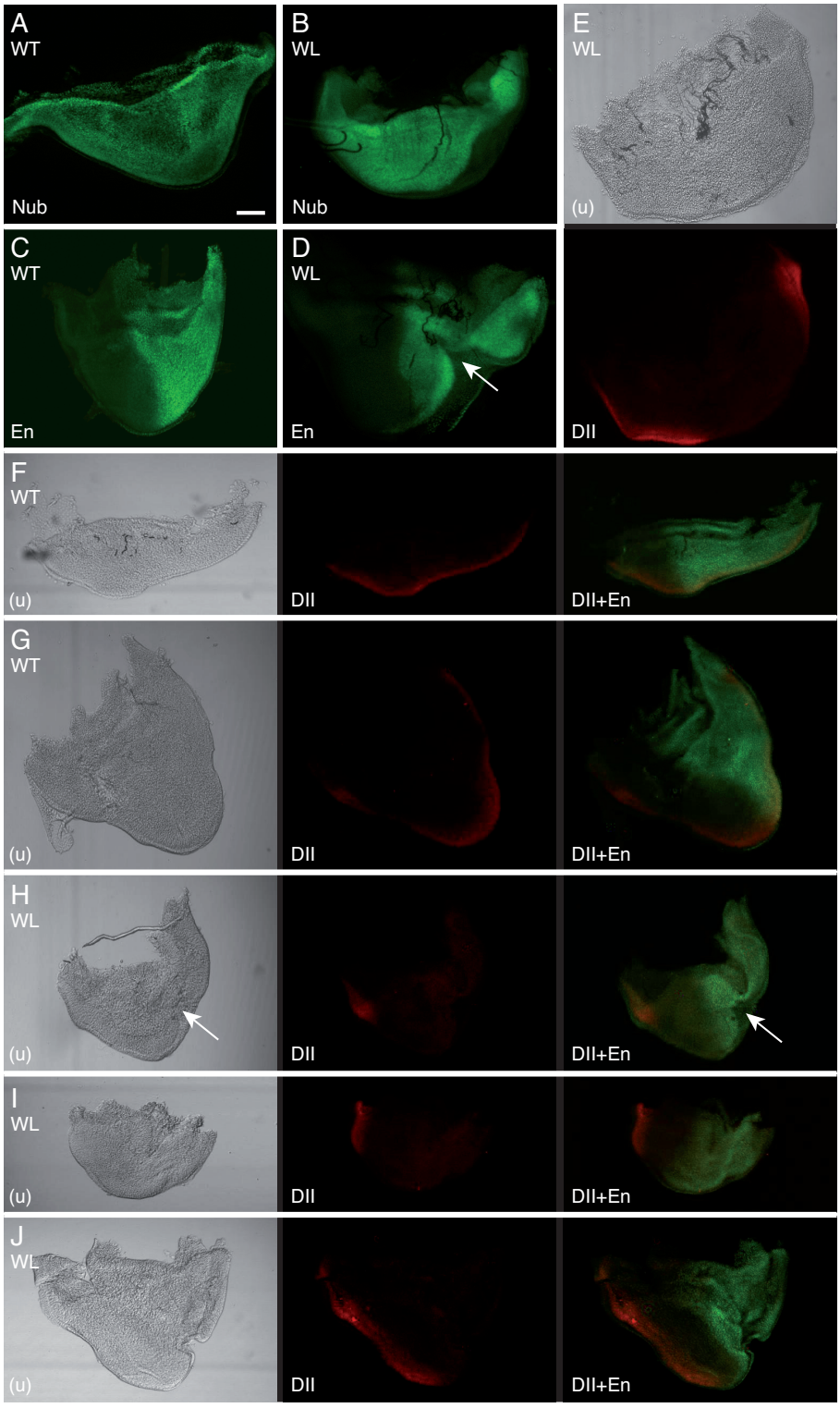
Figure 2. Wing length in *A. bipunctata*. A. The arrows point to the outer (O) and medial (M) part of the left elytron of a wingless individual. **B.** Regression of maximum hind wing on elytron length (mm) of wildtype (WT; y=1.489±0.344 + 1.467±0.099x) and wingless (WL; y=-0.416±0.154 + 1.065±0.060x) male and female adults of *A. bipunctata* shows that WL beetles have reduced wing lengths. The sexes did not significantly differ and showed no interaction with any other factor. In both phenotypes the lengths of elytra and hind wings are correlated, but in WL phenotypes hind wings are relatively more reduced than elytra. **C.** Maximum length of medial and outer elytron parts in wingless beetles. In both sexes, the outer part is significantly longer than the medial.

Histological examination of wing disc growth

Wing disc development in larvae of wildtype *A. bipunctata* corresponds to the description of wing disc development of ladybird beetles by Tower (1903). There is no difference between elytra and hind wing discs until the prepupal stage. Their development begins in L3 by thickening of the epidermal cells of the lateral meso- and metathorax (phase 1 in Fig. 3B; Fig. 3C). In late L3, the cells detach from the cuticle (apolysis). Invagination of the wing disc then takes place either just before or after moulting into L4 (phase 2). In the early-L4, the dorsal layer of the invaginated wing disc thickens and evaginates again to form the future wing (phase 3; Fig. 3D). The thinner ventral layer becomes the wing sac surrounding the wing disc. During the remainder of L4, the wing discs progressively extend ventrally (phase 4; Fig. 3E). The timing of these events varies among individuals by up to several days, but wing discs always increase rapidly in size during late-L4. In 15 of 16 winged individuals, wing length at this stage was over four times the width (phase 5; Fig. 3F). The wing sac then breaks open and the wing surface becomes folded (Fig. 3G). The elytra and hind wings differ in morphology at this stage as the elytron is characterized by a thickened dorsal cuticle.

In larvae of wingless beetles, wing disc growth similarly began in L3, but the remainder of the process was delayed (Fig. 3H-K). As a result, these beetles never had fully formed wings at the prepupal stage (Fig. 3L) but showed a variable degree of development. In none of the stages examined did wingless beetles contain features of apoptosis, such as haemocytes, picnotic cells, and empty spaces between cells.

Figure 4A and 4B show the variation in wing disc development for three wingless families, compared to the wildtype stock. All three wingless families lagged behind in their wing development throughout all stages of development, and the frequency distribution of larvae over developmental phases suggests that the delay in growth increased with time. The results also show differences in wing disc growth within the wingless phenotype. The differences in development between the three wingless families were not significant at earlier stages, but were by the mid- and late-L4 stage (mid-L4: χ_2^2=10.227, p<0.01; late-L4: χ_2^2=18.631, p<0.001). In the late-L4 stage, family A had more fully developed wings than the other families (compared to B: MWU=33.5, p<0.01; C: MWU=29.0, p<0.001), a pattern corresponding to the adult phenotypes (Table 1). Thus, family A, with the smallest lag in wing disc development had parents with the largest elytra, and adult offspring with the longest elytra of any wingless family (compared to B: MWU=416.5, p<0.01; C: MWU=11.5, p<0.001). In addition, adults of family B had longer elytra than family C (MWU=189.5, p<0.01), but this was not reflected in a significant difference in wing disc development in the histological samples of late-L4 larvae (MWU=80.5, p=0.545).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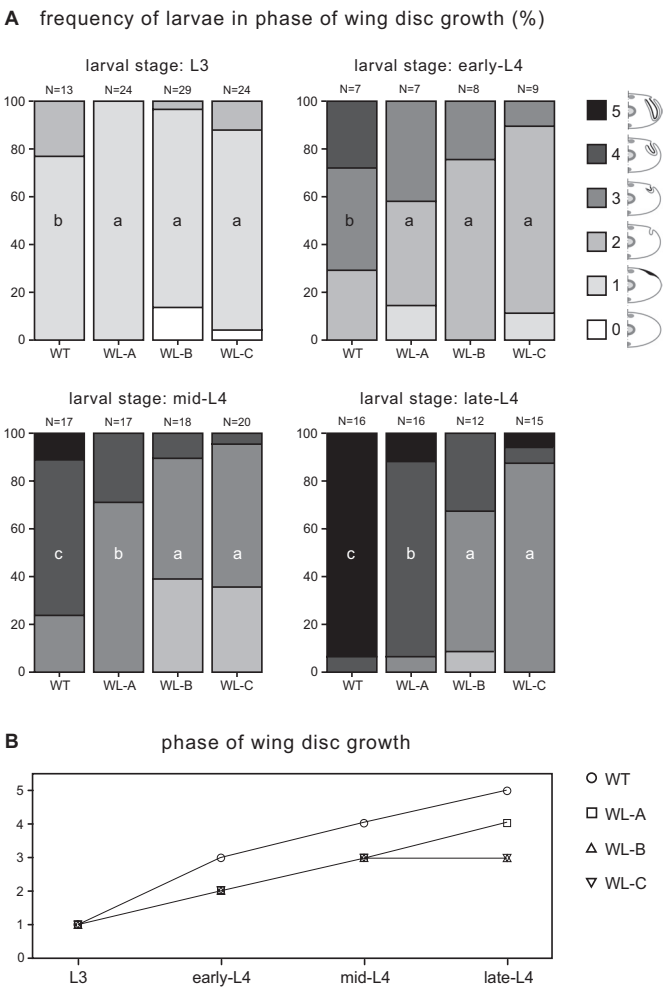

Figure 4. Variation in growth of wing discs in larvae of the wildtype *A. bipunctata* stock and three wingless families.
A. The frequency distribution of the phases of wing disc growth for four larval stages (L3, early-L4, mid-L4, and late-L4). Bars represent the wildtype stock (WT) and the three wingless families (WL-A to WL-C). Stacks represent the percentage of larvae in a particular phase of wing disc growth, the total number of larvae is shown above the upper stack. For each larval stage, significantly different distributions are indicated by different letters ($p<0.05$).
B. Summary of A with symbols representing the median phase of wing development for each group of ladybirds per larval stage.

Figure 5. Gene expression patterns in larval wing discs of wildtype (WT) and wingless (WL) *A. bipunctata* (see next page for Figure). Discs are displayed with the proximal side at the top and the distal tip at the bottom. If En was expressed, the anterior side is displayed at the left, and the (presumed) posterior side at the right. The scale is similar across images (scale bar in A. corresponds to 100µm). **A.** Expression of Nub (in green) throughout the wing tissue in a late-L4 WT. **B.** Similar Nub expression in a late-L4 WL. **C.** Expression of En (in green) in the posterior compartment in a late-L4 WT. **D.** Similar En expression in a late-L4 WL. This expression pattern demonstrates that the two lobes of the wing disc, as indicated by an arrow pointing at the notch separating them, do not correspond to the anterior and posterior compartment. **E.** Wing disc of a late-L4 WL unstained (u) (top), and with Dll expression (bottom, in red). Dll expression is missing distally, compared to WT discs. **F-J.** Panels each show the same wing disc unstained (u) (left), only with Dll expression (middle, in red), and with a double-staining of Dll and En (right, Dll in red, En in green). **F.** Disc of mid-L4 WT with Dll expressed along the entire wing disc margin. **G.** Similar Dll expression in late-L4 WT. **H-J.** Late-L4 WL larvae show that Dll expression is restricted to (part of) the anterior margin. **H.** Dll is restricted to a spot, located proximal anterior. The arrow indicates the separation between the two lobes, which do not correspond to the anterior and posterior compartment as revealed by En expression. **I.** Dll expression is somewhat extended, but still restricted to the proximal half of the anterior margin. **J.** Dll expression covers the anterior margin, but not the posterior margin.

Table 1. Degree of winglessness, classified from 0 (no elytra) to V (elytra covering more than ¾ of the body), in three wingless families of *A. bipunctata* used for histological sections. Values of the parents are given, together with the frequency distribution for their offspring. Significant differences ($p<0.01$) in those distributions are indicated by different letters in the last column.

family	degree of winglessness class		number of offspring per degree of winglessness class							sign
	father	mother	0	I	II	III	IV	V	total	
WL-A	0	II	2	5	1	6	2	0	16	a
WL-B	I	I	38	37	18	7	0	0	100	b
WL-C	0	I	7	1	0	0	0	0	8	c

Gene expression patterns in wing discs

The patterns of Nub, En, and Dll found in *A. bipunctata* larvae were identical in discs of elytra and hind wings. The transcription factor Nub is involved in specifying the early wing disc and plays a role in cell proliferation in *Drosophila* (Ng et al. 1995), while it occurs throughout the prospective wing. As expected, the same pattern was found in wildtype (Fig. 5A) and wingless ladybird beetles (Fig. 5B).

The transcription factor En plays a role in establishment and maintenance of the AP compartmentalization of the insect wing, and is located throughout the posterior compartment in other species of insects (Cohen 1993; Carroll et al. 1994; Abouheif and Wray 2002; Tomoyasu et al. 2005). It might be involved in development of wingless *A. bipunctata* since their wings are often partly truncated on the posterior side. Expression of *en* is also disrupted in wingless castes of several ant species (Abouheif and Wray 2002; Bowsher et al. 2007). En was found in both wildtype (Fig. 5C) and wingless ladybird phenotypes (Fig. 5D), and is indeed confined to one part (the posterior) of the wing discs. We further used this expression pattern to examine whether the anterior and posterior compartments might correspond to the two wing "lobes" often found in wingless *Adalia* (Fig. 1B,C,H,J-L). The "lobed" shape of larval wing discs occurred predominantly in wingless beetles but, surprisingly, also in some winged beetles. In all lobed discs, En covered the smallest lobe completely, and extended into the larger lobe (N=10, Fig. 5D and 5H, right panel).

Because wingless morphs of *A. bipunctata* lack the distal part of both pairs of wings, including the distal wing margin, we were interested in expression of the gene *Dll*. The transcription factor Dll controls normal cell differentiation of the wing margin in *Drosophila* (Campbell and Tomlinson 1998) and is found in wing margins of butterflies (Carroll et al. 1994; Brakefield et al. 1996). In wildtype beetle larvae, Dll was found along the entire margin of the wing discs in both developmental stages examined (Fig. 5F,G). In contrast, in late-L4 larvae of wingless beetles, *Dll* expression was incomplete (Fig. 5E,H-J), with Dll mostly confined to the proximal half of the anterior margin (Fig. 5H,I). In a few

cases Dll was extended to cover more of the anterior side (Fig. 5J) or showed an additional spot at the proximal side of the posterior margin (Fig. 5E). In the few samples of mid-L4 larvae of wingless beetles, Dll was not detected.

Discussion

Mode of adult wing reduction

We have shown that natural wingless phenotypes of the ladybird beetle *A. bipunctata* have two pairs of wings truncated (Fig. 1), which is unusual in two ways. First, hind wings are usually reduced in wingless beetles, but elytra are not. Elytra may have become consolidated, rigidly locked, or rounded after loss of flight (Smith 1964; Crowson 1981), but reduction has only been described for brachypterous rove beetles (Thayer 1992), in several species of brachypterous *Ptiliidae* (Dybas 1978), and in blister beetles (Crowson 1981). Elytra make up such a substantial percentage of the body weight that not having to produce them, or degrading them after they have been formed, is likely to save energy that could be allocated to fitness-related traits (e.g. Zera and Harshman 2001; Lorenz 2007). However, the loss of the elytron may be rare because it is constrained by its protective functions (Crowson 1981).

Second, the “lobed” wing shape typical of wingless adults has to our knowledge not been described for other wingless insects. However, some ant species have workers possessing vestigial wings asymmetric in size of the anterior and posterior compartment (Abouheif and Wray 2002). Using a modelling approach, Nahmad et al. (2008) found that the posterior compartment was likely to be more reduced because of asymmetrical growth, and suggested this was a consequence of the distribution of the Decapentaplegic morphogen, which is predominantly present in the anterior wing part and is required for cell growth.

Developmental mechanisms underlying reduced wings

Two developmental processes have been identified to regulate alternative wing morphs in insects: heterochrony of development and degeneration of wing tissue through apoptosis. These processes may occur in combination and at various stages of wing morphogenesis. For example, wing disc growth is arrested during larval stages in minor workers of the ant *Pheidole megacephala* (Sameshima et al. 2004), and in wingless females of the moth *Eumeta variegata* (Niitsu 2003). Degeneration occurs in nymphal stages of the pea aphid (Ishikawa et al. 2008), in pupae of major workers of *P. megacephala* (Sameshima et al. 2004), and in wingless females of *Diacamma* ants (Gotoh et al. 2005) and of several moths (Niitsu 2001; Lobbia et al. 2003).

Although wing polymorphism has been extensively documented in beetles (e.g. Darlington 1936;

Smith 1964; Dybas 1978; Hammond 1985), the mechanisms of wing reduction in this order are not well understood. In the few documented cases, wing cell degeneration took place in the pupal stage of wingless morphs (Smith 1964, and references therein). In contrast, we find that that freshly pupated wingless *A. bipunctata* already possess reduced wing cases, and that the extent of this reduction is correlated with the eventual reduction of the adult wings (Fig 1B-D). Therefore, wing reduction is probably the result of processes occurring earlier in development. This is confirmed by our histological examinations of wing disc growth in larvae. Cross sections of larval instars of *A. bipunctata* show that the wing discs of wingless morphs follow a similar pattern of growth to those of wildtype beetles, but with a time lag. At the time of pupation, they have substantially smaller wing discs (Fig. 3). Although hind wings are generally more reduced than the elytra in the adult beetles (Fig. 2B), the two pairs of wing discs show a similar growth schedule. This is perhaps because of processes taking place in the pupa, such as expansion of the wing tissue (Smith 1964). The overall pattern suggests that heterochrony, and, more specifically, a change in growth rate of the wing disc, is involved in the process resulting in wingless beetles. However, this cannot explain the distal truncation of the wing discs typical of the wingless morph of *A. bipunctata*, since there is no indication from any system that the insect wing is patterned in a regular proximal-to-distal sequence. Yet, it is unlikely that apoptosis plays any role, since we could not detect any features of this mechanism. It is possible that distal parts are missing from the fate map in wingless beetles, resulting in smaller, incomplete wing discs.

Variation in wing reduction among ladybird families

Our laboratory population of wingless *A. bipunctata* shows wide variation in the degree of winglessness. We examined this variation by making histological studies on three wingless families. The degree of winglessness typical of each family (Table 1) was generally correlated with the size of the wing discs in the larvae (Fig. 4). Overall, the results suggest that wingless beetles have slower wing disc growth, and that variation in the degree of winglessness is associated with variation in the rate of wing disc growth. The differences found among the wingless families in wing disc growth corroborate that this process has a heritable component (Ueno et al. 2004).

A continuous variation in wing reduction has been observed only in a few previous cases (e.g. Darlington 1936). Natural beetle populations exhibiting variation in wing length typically exhibit dimorphism with little variation in the wingless morph (Darlington 1936; Smith 1964). Monomorphic wingless phenotypes appear to have regularly become fixed in evolution, whereas the cases with continuous variation may reflect transitory phases which sometimes progress to fixation in time (Den Boer et al. 1980). Our results support the idea that complete wing reduction could evolve gradually in *A. bipunctata* if favoured by natural selection. Indeed, artificial selection in the laboratory has effectively led to lines showing near fixation of such an extreme wingless phenotype (this thesis, chapter 5).

Conservation of insect wing development

We explored expression patterns of three genes (*nub*, *en*, and *Dll*) involved in insect wing development in wing discs of winged and wingless *A. bipunctata* (Fig. 5). In *Drosophila*, their products act as transcription factors each in a different major wing patterning pathway: Nub is in the body wall/ wing patterning pathway which differentiates body wall and wing tissues (Ng et al. 1996), En is involved in initiating the AP wing patterning pathway (Held 2002), and Dll acts downstream in the dorsoventral (DV) wing patterning pathway (Held 2002).

The expression patterns in winged *A. bipunctata* were in agreement with those found in other insect taxa, such as flies, butterflies and ants (Cohen 1993; Carroll et al. 1994; Ng et al. 1995 ; Brakefield et al. 1996; Abouheif and Wray 2002), as well as in the beetle *T. castaneum* (Tomoyasu et al. 2005; unpublished results Y. Tomoyasu). These results support the idea that wing development is regulated by highly conserved pathways throughout the holometabolous insects (Abouheif and Wray 2002; *Tribolium* Genome Sequence Consortium 2008).

The DV wing patterning pathway is a candidate pathway for the development of wingless morphs

Expression of *nub* and *en* in wingless ladybird beetles was similar to that in wildtype beetles, and therefore not associated with the wingless phenotype (Fig. 5A-D). *En* expression in the posterior compartment of the wing discs also showed that the two “lobes” often seen in wingless beetles do not correspond to the AP compartmentalization (Fig. 5D, H). In contrast, the expression of *Dll* differed between winged and wingless beetles. From *Drosophila* (Campbell and Tomlinson 1998) and *Tribolium* (unpublished results Y. Tomoyasu), it is known that *Dll* is involved in specifying margin structures. In wildtype *A. bipunctata Dll* is expressed similarly at the wing margin (Fig. 5F-G). In contrast, *Dll* expression in wingless ladybird beetles is restricted to the proximal anterior margin (Fig. E, H-J). Interestingly, this location corresponds to the part of the wings that is typically most fully developed in adult wingless beetles. These results suggest an association between *Dll* expression and the wingless genotype. However, although down-regulation of *Dll* by RNAi in late larval stages reduces wings in the beetle *T. castaneum* (unpublished results Y. Tomoyasu) and the ladybird *Harmonia axyridis* (personal communication T. Niimi), it seems unlikely that *Dll* itself specifies development of the wingless genotype in *A. bipunctata*. First, the truncation of distal parts of the wing tissue can extend well beyond the wing margin where *Dll* is expressed in *A. bipunctata*; in the *Dll*-down-regulated *T. castaneum* beetles, only the wing margin is absent (unpublished results Y. Tomoyasu). Second, beetles with a mutation in *Dll* or down-regulation of *Dll* have truncated legs and antennae (Beermann et al. 2001; Niimi et al. 2005, unpublished results Y. Tomoyasu), whereas this is not found in wingless *A. bipunctata*. Therefore, the observed pattern is more likely to result from changes in the regulation of *Dll*. Alternatively, from *Drosophila* mutants it is known that mutations in many genes in the DV wing patterning pathway can result in truncated

wings, including *apterous, Notch, vestigial, scalloped, Beaded, Beaded-Serrate, Beadex, cut*, and *rudimentary* (overview in Lindsley and Zimm 1992), and combinations of these can cause more severely truncated phenotypes (Jack and DeLotto 1992). An association mapping approach could help to examine those alternatives.

We have observed variation in the disturbed *Dll* expression within the wingless morph of *A. bipunctata* (Fig. 5E, H-J). We hypothesize that this variation is associated with the degree of winglessness in the adult morphs. This should be examined further in the future, for example by using families differing in degree of winglessness. If, in addition, a functional relationship were to be found between the location of Dll and the degree of winglessness in *Adalia*, variation in the location of gene expression may provide a developmental mechanism underlying gradual evolution of wing loss, as discussed previously. In contrast, Nahmad et al. (2008) suggested from a modelling approach that a gradual evolution of wing loss involves a single key regulatory gene suppressing wing development in a dose-dependent manner. It is not known whether the gene *Dll* can act in such a manner.

In the wingless castes of all ant species examined to date for gene expression patterns, the gene network underlying wing development was disrupted in the AP patterning pathway (Abouheif and Wray 2002; Bowsher et al. 2007) which acts largely independently of the DV pathway (Held 2002). Thus, *A. bipunctata* provides the first example of a natural wingless phenotype associated with disruption in the DV patterning pathway. We cannot exclude, however, that *A. bipunctata* and ants also share interruption points until more genes of both patterning pathways have been examined. We have also shown that the phenotype of wingless *A. bipunctata* is unusual among wingless insects. Together, these results suggest that even though the gene network regulating wing development is conserved in winged morphs, different developmental mechanisms can produce winglessness and might have been involved in the evolution of wingless morphs throughout the insect world. It remains to be investigated which changes on the molecular level are controlling the development of wingless morphs, and this should be examined in more species of insect. This will provide opportunities to sort out whether differences in the nature of winglessness, such as polyphenism versus genetic polymorphism, and adaptive phenotypes (as in aphids and ants) versus low frequency mutations segregating in natural populations (as in *A. bipunctata*), mirror differences in developmental processes.

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Ellyn Bitume feeding ladybirds with aphids