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Cryptic variation revealed - the genetic architecture of winglessness in the predatory ladybird beetle, *Adalia bipunctata*

Suzanne T. E. Lommen, Kees G. Koops, Bardo A. Cornelder, Peter W. de Jong, and Paul M. Brakefield

Abstract

Cryptic variation is standing genetic variation that does not contribute to the phenotypic range observed under standard conditions, but can be released under perturbing environmental or genetic conditions. We report on the genetic architecture of cryptic variation in wing length reduction in the ladybird beetle *Adalia bipunctata* (L.) which is only released in genetically 'wingless' morphs of this (otherwise winged) species. In this naturally wingless morph both pairs of wings are truncated. We established a wingless stock by outcrossing individuals carrying alleles for winglessness to wild-types from the same locality in The Netherlands. This stock, fixed for the recessive wingless allele, displayed wide continuous variation in the extent of wing reduction. Split-families reared at two temperatures revealed strong family-by-temperature interaction for this variation. Heritability was 0.64±0.09 at 19°C and 0.29±0.06 at 29°C. Artificial selection at 21°C demonstrated that the degree of wing reduction can be altered rapidly. A phenotype without any wing tissue, as well as one resembling the winged wild type, were obtained within a few generations by selection in opposite directions in two replicates. Analysis of frequency distributions in a pedigree covering three generations of selection demonstrated that the heritable component of the variation in wing reduction involves at least two polymorphic genes. We discuss the evolvability of this heritable cryptic variation in the wild, and its relation to the evolution of winglessness in other insect species. Finally, we argue that the manipulation of wing length can be exploited to improve biological pest control by wingless morphs of this ladybird beetle.

Keywords

Coleoptera: Coccinellidae, cryptic genetic variation, gene-by-environment interaction, modifier genes, insect wing development

Introduction

Cryptic variation

'Cryptic variation' is standing genetic variation that does not contribute to the range of phenotypes observed under standard conditions that natural populations experience, but can be 'released' and contribute to the phenotype under perturbing environmental or genetic conditions. The existence of such variation has long been recognized (reviewed by Schlichting 2008), and is common (e.g. Rutherford and Lindquist 1998; Sangster et al. 2008a). It has recently regained attention in relation to the developmental mechanisms that regulate its expression (reviewed by Rutherford 2000; Gibson and Dworkin 2004; Sangster et al. 2008b), and how it contributes to the evolvability of traits (Rutherford and Lindquist 1998; Rutherford 2003; Masel 2006; Le Rouzic and Carlborg 2008; McGuigan and Sgro 2009; Hayden et al. 2011).

Cryptic variation contributes to wing reduction in flightless morphs of *Adalia bipunctata*

Here, we describe the genetic architecture of cryptic variation in the two-spot ladybird beetle, *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae), that is revealed in a natural 'wingless' morph of this species (Marples et al. 1993). Wild-type beetles are fully winged and monomorphic in their wing length, with elytra (fore wings) covering the entire dorsal surface (Fig. 1). Their flight wings (hind wings) are folded underneath the elytra when at rest, but are nearly twice as long as the elytra when unfolded in flight (Lommen et al. 2009). 'Wingless' morphs are occasionally encountered in the wild (Majerus and Kearns 1989; Marples et al. 1993). They have been recorded from at least one population in The Netherlands (Marples et al. 1993), and five in the United Kingdom (M.E.N. Majerus, personal communication). Their elytra and flight wings are truncated (Lommen et al. 2009) so that they cannot fly. Although they may have some wing tissue, we will for ease, call them 'wingless'. This trait is determined by a major locus with the winged, wild-type allele being dominant over the wingless one (Marples et al. 1993; Ueno et al. 2004) (Fig. 1). Since only a few wingless specimens from the wild have been reported on in detail, the variation within the wingless phenotype in the wild is unknown. However, several wingless stocks have been established by breeding from a single wingless individual taken from the wild near Utrecht in The Netherlands and crossed with wild types from the same locality. In each case, the wingless F2 individuals exhibited wide variation in the extent of the wing reduction (Marples et al. 1993; Ueno et al. 2004; Lommen et al. 2009). This variation is continuous from individuals lacking all wing tissue to ones missing only very small pieces at the wing tips (Fig. 1). This variation is associated with fitness traits since those with more marked reductions develop more slowly and have a shorter life span (Ueno et al. 2004). Earlier work suggests that the extent of wing reduction is a result of differences in the rate of wing development in the larval stages, and is regulated by both genetic and environmental

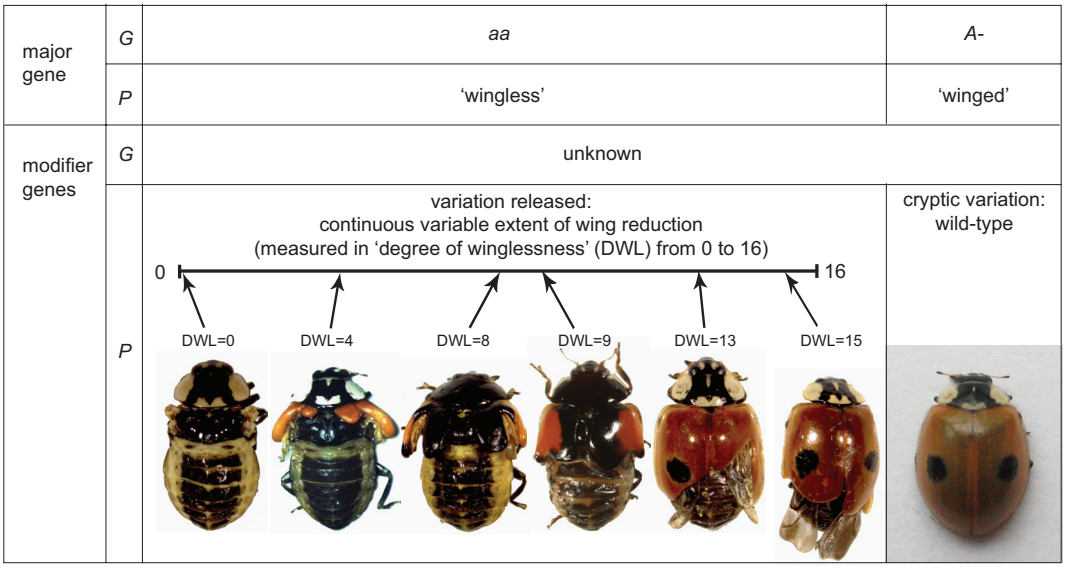


Figure 1. Genetic (G) and phenotypic (P) variation in wing morphology in *Adalia bipunctata.* The upper part of the graph covers the major gene regulating winglessness, the lower part shows the modifier genes regulating the extent of wing reduction in wingless morphs. This extent is expressed in the degree of winglessness of the elytra (DWL), a categorical measure with values ranging from 0 (no wing tissue) to 16 (elytra covering more than ¾ of the abdomen, see materials and methods for details). The pictures of the six *wingless* specimens represent examples of particular DWL categories. The middle two individuals are *melanic* colour morphs, whereas the others are *typical* morphs. A *wild-type* (winged) typical ladybird beetle is shown at the right.

factors (Ueno et al. 2004; Lommen et al. 2005; Lommen et al. 2009). Since all wingless beetles are homozygous recessive for the wingless trait, the heritable variation in the extent of wing reduction within wingless morphs is determined in the genetic background that reflects the standing genetic variation in the almost completely winged wild population. Thus, variation in the extent of wing reduction is cryptic in the wild-type phenotype, whereas it is released in the wingless phenotype.

The significance of this cryptic variation

The significance of this cryptic variation in natural populations of *A. bipunctata* is unclear, since wingless individuals are scarce and the potential for pleiotropic effects on fitness traits in wild types is unknown. Nevertheless, the variation in the extent of wing reduction in wingless adults is of interest in this system for several reasons. Firstly, winglessness is a derived trait that is common in beetles (e.g. Darlington 1936; Smith 1964; Dybas 1978; Hammond 1985), and in many other insect groups (Wagner and Liebherr 1992; Roff 1994a). The mechanisms of development and evolution of wing reduction are, however, diverse and not fully understood (e.g. Roff 1994b; Abouheif and Wray 2002; Brisson et al. 2010). Typically, wing polymorphic populations exhibit discrete winged and wingless phenotypes that each show little variation in wing morphology. Only in a few cases

has variation in the extent of wing reduction within the 'wingless' morph been recorded in nature. These include examples in beetles (Darlington 1936; Den Boer et al. 1980; Desender 1989), and stoneflies (Plecoptera) (McLellan 1999). Such variation might provide a key to understanding instances of the evolution from entirely winged populations to those including wingless morphs. Secondly, there is interest in the use of wingless morphs of ladybird beetles as natural biological control agents of insect pests in agriculture (Lommen et al. 2008; 2013). Understanding the basis of variation within the wingless morph, including the potential for its manipulation, may help to optimize this morph for its application in biocontrol.

In this paper, we investigate in detail the genetic architecture of variation in the degree of wing reduction in wingless *A. bipunctata*. We quantify the heritability of the degree of wing reduction, demonstrate that we can change this rapidly by artificial selection, and examine genetic models that can explain observed patterns of segregation using a pedigree approach.

Materials and methods

Beetle laboratory stock

A laboratory stock of wingless *A. bipunctata* was established from individuals collected in the wild in Utrecht in The Netherlands on several occasions. Beetles were collected from trees or shrubs. Two individuals were found to bear copies of the wingless allele, one homozygote and one heterozygote. The three founding wingless alleles are assumed to be identical by descent (see further below). The wingless allele was fixed in the laboratory stock by outcrossing these beetles to more than 100 wild types and then selecting only wingless phenotypes as parents in the large F2. This stock was then used for all experiments. It is maintained at 20.5°C ± 1.5°C, RH 65% ± 5%, and 16L:8D. Larvae and adults are fed with *Ephestia kuehniella* Zeller eggs, with adults receiving a supplement of flower pollen (De Clercq et al. 2005).

Measuring wing reduction

We used the measure 'degree of winglessness' (DWL) to quantify the continuous variation in wing reduction of wingless *A. bipunctata* (see also Lommen et al. 2005). The degree of the truncation of the elytra is closely correlated with that of the flight wings (Lommen et al. 2009). The latter are not visible when folded beneath the elytra and, therefore, DWL is based on the length of the elytra alone. DWL indicates the length of the truncated elytra relative to the maximum length as would be recorded in the wildtype where the elytra cover the entire abdomen. Because the left and the right elytron, and even the medial and outer half of a single elytron, may be truncated to different extents, the length of each of these four halves is scored separately. The length of each

of the four elytral sections was estimated by eye and assigned 0 (no tissue visible), 1 (only a small bud, not extending beyond ⅛ of the maximum), 2 (up to ¼ of the maximum), 3 (more than ¼ and up to ½ of the maximum), 4 (more than ½ and up to ¾ of the maximum), or 5 (more than ¾ of the maximum). The sum of these four scores is an individual's DWL, with a minimum value of 0 and a maximum of 20, with higher numbers corresponding to increased elytron length (Fig. 1). This method is highly repeatable (Lommen et al. 2005). However, here it proved problematic for statistical analysis because the range of DWL categories is not symmetric: more than half of the DWL categories represent tissue covering up to only half of the maximum tissue length. Therefore, we modified the calculation of DWL by combining score 1 (only a small bud, not extending beyond 1/8 of the maximum) and 2 (up to ¼ of the maximum). As a result, the new summed DWL value (the sum of the four scores) became a symmetric range from 0 to 16 (Fig. 1). Individuals with a DWL of 0 have no elytral tissue, whereas, a DWL value of 16 was assigned to beetles with all four elytral parts extending beyond ¾ of the maximum elytral length (note that morphological variation remains within this latter category).

Heritability experiment

A series of families of wingless *A. bipunctata* were bred to estimate the heritability of DWL by parent-offspring regression. In addition, the offspring of each family were split and reared at two different temperatures to examine environmental effects. Three-week old virgin adults from the stock were used to set up pairs. We first selected individuals with different DWL values to maximize variation in wing reduction in the parents. We then formed pairs using males and females with nearly equal DWL values. In this way the range of mid-parent values was maximized to increase the reliability of the heritability estimate (Falconer and Mackay 1996). Pairs were kept in round Petri dishes (Ø 55 mm) with paper lined in the cover to stimulate egg-laying under our rearing conditions. These papers were collected and replaced every 2-3 days over a period of 12 days. Collected papers with eggs were moved to a clean dish. Hatched first instar larvae were again moved to a clean dish and reared in groups of up to 10 until moulting. After moulting, the second instar larvae were randomly assigned to an incubator with one of two rearing temperatures, 19°C or 29°C, and within which their position was regularly changed. Histological examinations show that the formation of wing structures does not begin until this larval stage or later (Lommen et al. 2009). Emerged adults were frozen at -20°C before their sex and DWL were determined within several weeks. We selected for analysis those families with at least five offspring at each rearing temperature (N = 41 families, median = 15 and 12 offspring at 19°C and 29°C, respectively).

Statistical analysis used the package R, version 2.6.1 (R Development Core Team 2007). The mid-offspring values were represented by the median DWL of each family to correct for non-normal frequency distributions. We pooled sons and daughters since sample sizes per sex were small

whereas the variation among offspring was large, and because earlier work revealed no differences between the sexes (Lommen et al. 2005). The heritability experiment was analysed using linear models fitted by least square methods using weighting by the number of offspring. In the full model, family, temperature, and their interaction term were specified as fixed factors. We tested the significance of the interaction by removing this term from the model and comparing the models by an F-test. The heritability of DWL was then estimated for each rearing temperature separately as the mid-offspring on mid-parent regression.

Artificial selection experiment

An artificial selection experiment was performed to estimate the realized heritability of DWL in our stock at 20.5°C and 65% RH. We selected in both upward and downward directions using two replicates. Generation 0 (G0) was derived from the wingless stock after artificial selection for increased fecundity over ten generations (S. T .E. Lommen, K. G. Koops, P.W. de Jong., and P. M. Brakefield, unpublished data). We randomly split this population in half to create the two replicate lines: 'C' (original N=1136 virgin beetles) and 'D' (N=1126). For each replicate, we selected 60 males and 60 females with the most extreme values in each direction: 'L' and 'H' for downward and upward selection, respectively (yielding lines: 'CL', 'CH', 'DL', and 'DH'; Table 1). For each line, the selected adults were randomly distributed into three breeding groups, each of 20 males and 20 females, at an age of 6-26 days when most beetles are sexually active (Hemptinne et al. 2001). Breeding groups were kept in Petri dishes (120 mm x 120 mm) with paper folded like a harmonica to stimulate egg-laying. Eggs were collected every 2-3 days over two weeks (except for G4, over four weeks) and reared to adulthood. Newly emerged adults were collected every 2-3 days and housed individually in small dishes (Ø 55 mm) until after determination of their sex and DWL to ensure that all adults remained virgin until after selection. Occasionally when insufficient offspring were bred, fewer than 60 adults per sex were collected (as in G2 and G3 of line CL; Table 1). When the most extreme DWL value in the direction of selection contained more than 60 adults per sex, all of them were selected as parents (Table 1). Selection was continued for four generations, after which an extra round of selection was performed for the upward selected lines to check whether the potential remained for a further response towards an elytral phenotype even closer to that of the wild type. The phenotype of beetles with a DWL of 16 for a subset of the G4 (see Table 1) and for the entire G5, was described in more detail by recording whether the elytra covered the full length of the abdomen. All beetles selected as parents in G4 for the extra round of selection had a DWL of 16 and were of this latter phenotype.

To analyze the results, we first tested whether DWL differed between the sexes for each selection line at each generation. We used a Wilcoxon Signed Rank Test with continuity correction, adjusting the alpha with a Holm-Bonferroni correction. Since no significant differences were detected, we

Table 1. Numbers of bred and selected adults per generation for each selection line

replicate	direction of selection	line		number of adults in generation					
				0	1	2	3	4	5
C	downward	CL	bred:	1136[a]	464	42	35	156	NA
			selected:	120	133	39	32	NA	NA
C	upward	CH	bred:	1136[a]	552	785	606	519[b]	881
			selected:	120	120	120	160	120	NA
D	downward	DL	bred:	1126[c]	329	292	133	99	NA
			selected:	120	122	149	102	NA	NA
D	upward	DH	bred:	1126[c]	710	455	547	777[d]	781
			selected:	120	119	120	160	120	NA

NA=not applicable
[a] CL and CH were selected from the same group of G0 adults
[b] for a subset of 320 beetles, elytra of those with DWL=16 were described in more detail
[c] DL and DH were selected from the same group of G0 adults
[d] for a subset of 577 beetles, elytra of those with DWL=16 were described in more detail

pooled the sexes for further analysis. A comparable procedure was followed to examine differences in DWL between the low and high line of each pair of replicate lines in each generation. We then estimated the realized heritability of the DWL for each selection line as the slope of the regression of the cumulative response to selection (median offspring value - median of the total parental population) on the cumulative selection differential (median of the selected parents - median of the total parental population). We calculated the mean realized heritability for each direction of selection by averaging the values of the two replicates. A heterogeneity G-test (Sokal and Rohlf 1995) was used to test whether the proportion of beetles with a DWL of 16, and with elytra covering the full length of the abdomen, had changed after the extra round of selection in the upward selection lines. Finally, a 1-tailed paired t-test was used to test the hypothesis that the average number of offspring per selected adult was higher for the lines selected in the upward than in the downward direction for each of the replicates.

Pedigree analysis

To study the genetic architecture of wing reduction in wingless *A. bipunctata* in more detail, we created a pedigree over four generations, and then examined whether genetic models could be fitted to the data. We used G4 of each of the four selection lines to set up the pedigree as shown in Figure 5a. Each family was bred from a pair of virgin beetles. We first set up pairs within each selection line with similar DWL values (P). In the next generation (F1), we crossed individuals descending from opposite selection directions within each replicate (i.e. CL x CH and DL x DH). In addition, we created inter-line crosses between the replicate high lines (CH x DH). Offspring from

these crosses (F2) were then interbred within their own group to produce the F3 families (parents always came from different families to minimize inbreeding). We determined the sex and DWL of all individuals raised and only included families with more than 20 offspring in the analysis (except for one smaller, but essential, family) yielding a total of 96 families. Since very rapid responses to artificial selection occurred (see results), we hypothesized that DWL is regulated by a small number of genes and begun our analysis using a model of one polymorphic gene.

Data archiving

The data sets resulting from each of these experiments will be deposited in the public, digital archive Dryad (www.datadryad.org).

Results

Heritability experiment

The degree of winglessness (DWL) of the parents ranged from 0 to 12, since pairs with higher DWL values produced insufficient offspring, and were excluded from the analysis. There was a significant family-by-temperature effect ($F=9.98$, $p<0.01$). Raising larvae at higher temperatures usually yielded lower median DWL values (Fig. 2), with this pattern being observed in 35 of 41 families. The mean heritability was estimated as 0.64 ± 0.09 (SE) at 19°C ($F_{1,39}=53.35$, $p<0.001$, $R^2=0.58$) and 0.29 ± 0.06 (SE) at 29°C ($F_{1,39}=23.00$, $p<0.001$, $R^2=0.37$) (Fig. 2).

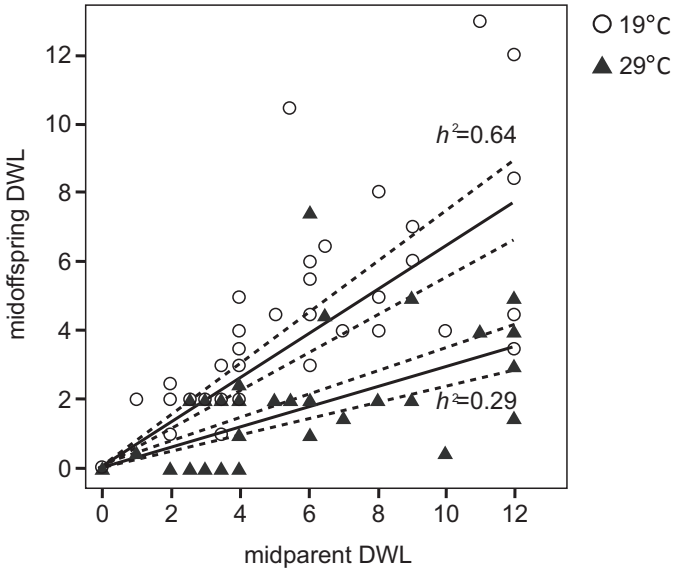


Figure 2. Heritability of DWL. The degree of winglessness (DWL) plotted as the midoffspring on midparent values for split families raised at two temperatures. The slope of the regression lines (shown between 95% confidence intervals) indicates the heritability (h^2) of DWL estimated for each temperature, and significantly differs between the two temperatures.

Artificial selection experiment

Males and females of G0 did not significantly differ in DWL (N_{males}=1130, $N_{females}$=1132, W=615508.5, p=0.118). An absence of a sex effect was also shown after the population was split into replicates and selected for one generation (CL: W=28037.5, p=0.337; CH: W=35214, p=0.362; DL: W=12799, p=0.3752; DH: W=67930, p-value=0.070), and in all of the subsequent generations of all four selection lines (not shown). A rapid response to artificial selection on DWL was observed in the replicate lines in each direction (Fig. 3). In both replicates, the upward and downward lines differed significantly from each other in DWL after only a single generation of selection (C: W = 17831.5, p<0.000; D: W=13480, p<0.000). The response to selection continued until in both downward selected lines, the median DWL reached the minimum possible value (DWL=0) after only two generations, whereas that of the upward selected lines both took four generations to reach the maximum score (DWL=16). Corresponding realized heritabilities were 0.72 ± 0.07 and 0.61 ± 0.03 for downward and upward selection, respectively (Fig. 4).

Although the majority of the beetles in the upward direction had reached this maximum score after four generations of selection, the proportion of individuals with elytra covering the full length of the abdomen was only 48% and 35% in CH and DH, respectively. It is noteworthy that the majority of these latter beetles remained distinct from wild types because of irregularities in the three-dimensional shape of their elytra.

After the extra selection round of selection in which all parents had elytra covering the full length of the abdomen, the proportion of individuals with a DWL of 16 significantly increased from 65% to 70% in replicate CH (G_H=3.845, p<0.05) and from 50% to 71% in DH (G_H=68.216, p<0.001). The proportion of individuals with elytra covering the full length of the abdomen did not change significantly in line CH (G_H=2.297, p=0.130; 48 to 43%), whereas it increased from 35% to 46% in line DH (G_H=16.493, p<0.001).

In line D, the number of offspring produced was significantly higher in the upward than in the downward direction of selection (t=5.36, df=3, p<0.01). The same trend occurred in line C but was not significant (t=1.33, df=3, p=0.138).

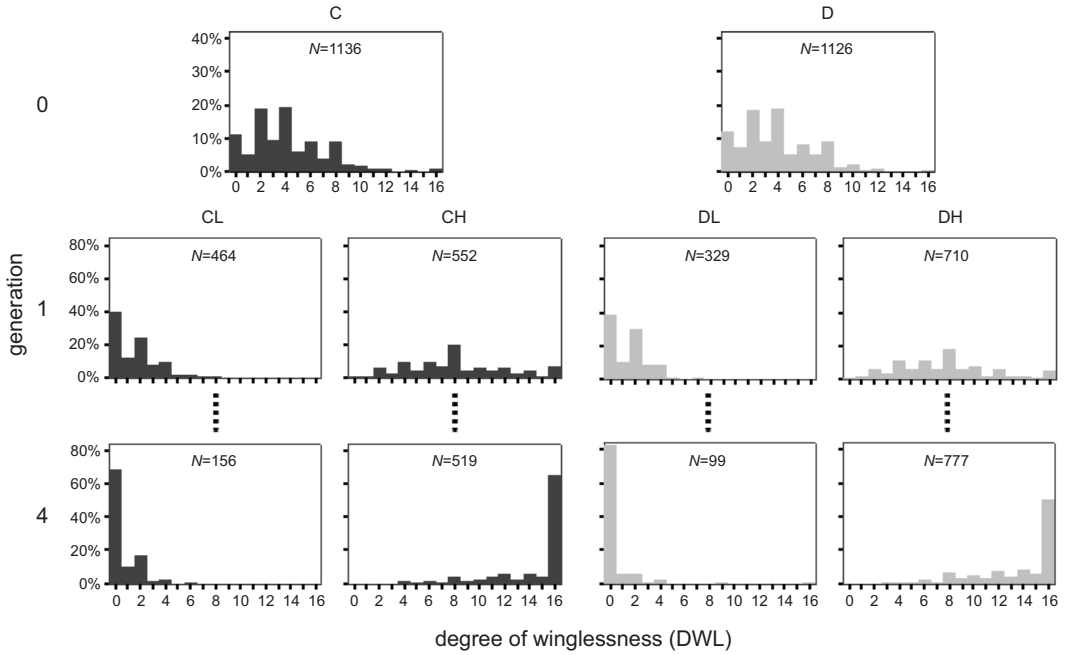


Figure 3. Artificial selection for extreme DWL. Frequency distributions of two replicate lines (C and D) artificially selected in downward (L) and upward (H) directions for degree of winglessness (DWL). Bars in histograms represent percentages for each DWL class, whereas total sample sizes are indicated for each histogram. The initial subpopulations (generation 0) are shown, together with the populations one, and four generations after selection began. There is a strong response to selection, and after four generation the majority of the beetles has reached the maximum value in the selected direction. The replicates show a similar pattern.

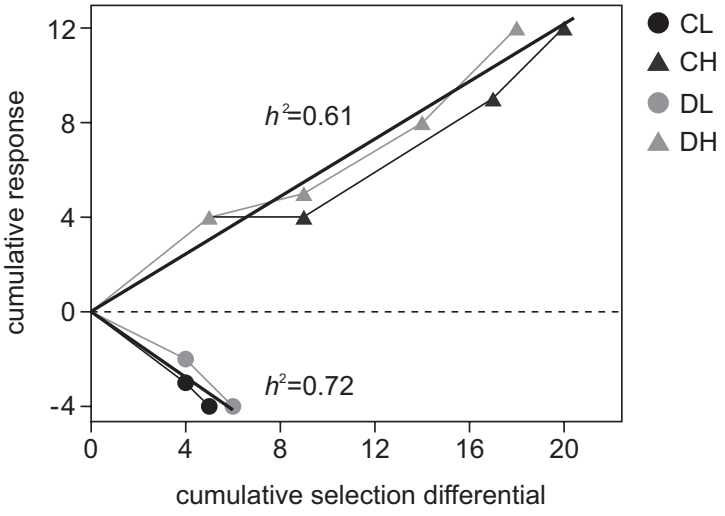


Figure 4. Realized heritabilities of DWL. Cumulative response to artificial selection as a function of the cumulative selection differential for both replicates (C, D) selected in upward (H) and downward (L) directions for degree of winglessness (DWL). Mean realized heritabilities (h^2, thick solid lines) are given for each selected direction.

Pedigree analysis

We examine here a representative part of the entire pedigree that comprises 15 families over three generations (data from other parts of the pedigree yielded consistent interpretations; the full data set is available on request). Using 15 families (Fig. 5b), we first demonstrate that variation in DWL is unlikely to be regulated by a single polymorphic locus with two alleles before moving on to more complex models. In the context of a single-gene, bi-allelic model we can make several general predictions with respect to the parental genotypes used to found the pedigree from the selection lines at G4 (generation P). Given that the lines showed progressively less variation with each generation of selection, and that those selected in opposite directions displayed very little overlap in phenotypic distribution after four generations (Figure 3), we assume that individuals from opposite selection lines are near fixation for alternative alleles at this single gene, and that the majority of them are homozygous. Since the estimated heritabilities of DWL were high, variation in wing reduction appears to be mainly additive and we assume no dominance or epistatic interactions. Under these assumptions, we can predict that the frequency distributions of DWL of the offspring are: 1) different for F1 families from lines selected in opposite directions; 2) similar for F1 families from lines selected in similar directions; 3) equal across all F2 families, regardless of parental origin, and consist exclusively of heterozygotes; and 4) equal across all F3 families which would constitute each of the three possible segregating phenotypes, and would therefore have a wider range of phenotypes than the F2.

We can then compare these predicted phenotypic distributions to those observed in the 15 families shown in Figure 5b and all descended from lines DL and DH. Phenotypes of the parents (DWL values) are given at the top of each histogram (note that all founding parents had extreme DWL values of either 0 or 16, increasing the likelihood that they were homozygote under the assumptions above). Prediction 1 fits the results since F1 families from opposite lines indeed differ in their frequency distributions (DL: family 2 and 4, versus DH: family 1, 3, and 5). However, the remaining predictions do not fit the pedigree. Prediction 2 is rejected because F1 families from DH display variation in the frequency distribution (e.g. family 3 versus 5). Prediction 3 does not hold because the distributions are variable across families within generation F2 (compare families 6-7 with 8-10). Prediction 4 does not fit the observed data because distributions of F3 families differ from each other (families 11-15), do not cover a wider range than the F2 families, and do not all include all three possible segregating phenotypes under the bi-allelic model (e.g. family 13 lacks high DWL values). Even allowing for heterozygosity in some founding individuals to explain variation across the F1 families, the model cannot explain patterns of variation in the F2 and F3 families. Hence, we reject a single-gene, bi-allelic model for the control of wing reduction.

We then explore a model of a single gene with multiple alleles, and also allow founding parents to be heterozygous rather than homozygous. However, this model fails to explain the absence of some

phenotypes in F2 and F3 (e.g. families 6, 7, and 13 lack high values). Thus, the pedigree reveals that a single polymorphic gene, regardless of the number of alleles at this locus, cannot explain the observed variation of wing reduction within the wingless morphs, and we conclude that variation in wing reduction is regulated by more than one locus (i.e. polygenic). With this conclusion, we implicitly reject the possibility that variation is explained by an allelic series of several wingless alleles at the major wingless locus.

A model with two bi-allelic genes with full dominance but without epistatic interactions also failed to explain the data because two parents with low values of DWL (corresponding to homozygote recessives at one of the two genes) were able to produce the full range of phenotypes (including phenotypes corresponding to dominant alleles on this gene) (e.g. family 14, Figure 5) . Therefore, we also reject this model and conclude that a more complex one is required. Such a model could involve more genes, more than two alleles at some genes, or epistatic interactions (note that reciprocal crossings give no evidence for paternal or maternal effects, e.g. family 6 versus 7).

Discussion

The genetic architecture of wing length variation in *A. bipunctata*

Our results show that the variable extent of wing reduction in wingless morphs of *A. bipunctata* is highly heritable (Fig. 2 and 4), and that there are gene-by-environment interactions (Fig. 2). The degree of winglessness changed rapidly under artificial selection over four generations (Fig. 3). These results suggest that few loci with large effects are involved. The analysis of the pedigree (Fig. 5) shows that the inheritance of the extent of wing reduction in our stock is polygenic, and that these genes are likely to be multi-allelic. None of the experiments or the pedigree indicates any genetic linkage between these genes and sex.

Thus, we can now present a more complete model of the genetics of winglessness in this species. This is likely to involve at least three polymorphic loci on the autosomes regulating wing morphology: one determining the wingless status (Marples et al. 1993, upper part of Table 2), and at least two multi-allelic modifier loci that are affected by the environment and regulate the variation in the extent of wing reduction within the wingless phenotype (this paper, lower part of Table 2).

In the artificial selection experiment, an extra round of selection in the upward direction resulted in an increased proportion of individuals in the highest category, DWL=16, but only one of the two replicates resulted in more beetles with elytra covering the full length of the abdomen. This suggests that at least one polymorphic modifier locus had not yet reached fixation.

Since all wingless individuals in our experimental stock are homozygous recessive at the wingless locus, our results confirm that the expressivity of the wingless mutation is dependent on the

Table 2. A genetic model suggested for natural wing polymorphism in *A. bipunctata*. The table provides the number of genes involved (N), the number of alternative alleles per gene, the position of the genes, the heritability and expression of the trait regulated. The upper part of the table provides details on the major gene regulating winglessness, the lower part on modifier genes associated with variation in the expression of this trait.

	regulating	N	alternative alleles	position	heritability	expression	sources
major gene	wingless-ness	1	2	on an autosome, linked with gene for melanism	1.0	**dominance:** 'winged' completely dominant over 'wingless' **epistasis:** 'winged' allele shows full penetrance and dominant epistasis to the modifier genes	Marples *et al.* 1993; Lommen et al. 2012
modifier genes	extent of wing reduction	≥2	≥2 at each locus	on autosomes	**parent-offspring regression:** - at 19°C: 0.64±0.09 - at 29°C: 0.29±0.06 **realized h^2** at 21°C**:** - upward: 0.61±0.03 - downward: 0.72±0.07	**conditional:** - when major gene has at least 1 'winged' allele: no visible phenotype (cryptic) - when major gene is homozygous for 'winglessness': expression dependent on *G*x*E* interaction (variation 'released')	Ueno et al. 2004; Lommen et al. 2005; this paper

genetic background (Ueno et al. 2004; Lommen et al. 2005; 2009). The latter reflects the standing genetic variation at the field location, which has no visible phenotypic effect on wing morphology in the winged phenotypes (Lommen et al. 2009). This cryptic variation could have evolved by the accumulation of neutral mutations that scarcely, if at all, affect traits under selection in the wild type. Alternatively, it could have positive pleiotropic effects on such traits under selection, and have evolved by stabilizing selection on these traits (e.g. Duveau and Felix 2012). However, the substantial amount of heritable variation for the degree of winglessness, as demonstrated in our study, suggests that this variation is neutral with respect to fitness traits. Genetic canalization, where wing development is robust against genetic mutations, may be a mechanism explaining its cryptic nature (Waddington 1957; but see Hermisson and Wagner 2004; Zhang 2008).

Although cryptic variation is hard to detect, especially in nature, this phenomenon has been recognized in several other complex traits in model organisms. Examples include sense organs and body appendages of *Drosophila melanogaste*r (Gibson and van Helden 1997; Polaczyk et al. 1998; Atallah et al. 2004; Dworkin et al. 2009), diseases in mice and humans (Nadeau 2001), and sexual characters in *Caenorhabditis elegans* (Milloz et al. 2008; Chandler 2010). Recent studies in these organisms show that background effects can have substantial phenotypic consequences as large as those of known mutations in major genes (Gibson et al. 1999; Atallah et al. 2004). Our artificial

selection experiment similarly demonstrates that the wingless phenotypes in *A. bipunctata* span the range from those without any wing tissue to those indistinguishable from the wild type.
Our results confirmed that there is a family-by-temperature effect on the extent of wing reduction in *A. bipunctata*, providing evidence that the genetic background and the environment interact to produce the phenotype. Some other studies have also revealed cryptic genetic variation that interacts with the environment highlighting how a complex interplay between these factors may regulate the development of complex adult phenotypes, and that understanding such interactions will be crucial in explaining phenotypic diversity (Atallah et al. 2004; Chandler 2010). A challenge for future research is to examine the evolution of cryptic variance, and its consequences for the evolvability of traits in natural populations (Le Rouzic and Carlborg 2008; McGuigan and Sgro 2009).

Evolvability of the cryptic variation in the extent of wing reduction

Our artificial selection experiment demonstrates that the cryptic variation regulating the extent of wing reduction is evolvable in a laboratory environment. Wingless phenotypes showing little variation were obtained in each direction of selection. Thus, the downward direction yielded individuals consistently without any wing tissue, whereas a phenotype closely similar to the wild type resulted in the upward direction effectively masking the wingless genotype with respect to elytra morphology.

Restoration of the wild-type phenotype including flight ability

The extent to which the flight ability of beetles homozygous for the *wingless* allele can be restored remains to be investigated. Histological examinations of the flight muscles of wingless morphs did not reveal any reduction in muscle tissue (S. V. Saenko and S. T. E. Lommen, unpublished results), and wingless individuals were frequently observed to move their truncated wings up and down in a similar manner as wild types (S. T. E. Lommen and K. G. Koops, unpublished results). The flight wings of wingless individuals were usually reduced or malformed (Lommen et al. 2009), and the beetles were typically not capable of directed flight. However, observations on flight behaviour suggest that the potential for at least some flight activity was restored in a small proportion of wingless individuals after artificial selection in an upward direction (S. T. E. Lommen, K. G. Koops, P. W. de Jong, P. M. Brakefield, unpublished data). Thus, when five random samples of ten G4 beetles each from the upward selection lines were given the opportunity to fly up from vertical sticks surrounded by a water barrier, several individuals attempted to fly by opening their elytra and spreading their flight wings, but only a few of these managed to take off (of which only a single individual flew up substantially, more than a meter in height), and all of them quickly dropped down or landed in the barrier. In contrast, from one group of ten wild types, five flew up and away passing

the water barrier, and a further two flew up and landed in the water barrier. In another flight test where phenotypes with elytra both in length and in three-dimensional shape indistinguishable from the wild type were released in the air, only three out of 17 beetles flew in a directed manner, whereas 16 out of 18 wild types did.

Such evolution towards a wild-type phenotype has also been observed in the laboratory for the eyeless-4 mutant of *D. melanogaster* (Spofford 1956). Isogenic populations, homozygous recessive for the eyeless trait, displayed variable reduction in eye size which was negatively correlated with reproduction, and mean eye size moved towards that of the wild type eye size over several rounds of selective breeding. Several mechanisms could account for such a recovery of wild-type phenotypes including selection of modifier genes that diminish expression of the mutant phenotype through epistasis (Gibson and Dworkin 2004), selection of an epigenome that buffers the effect of the mutation in the major gene (Sollars et al. 2003; Johannes et al. 2008), or selection for modifiers that produce the wild type phenotype via another route in the genetic regulatory network (Tautz 1992; Kitami and Nadeau 2002).

Evolution of wing reduction in the wild

How likely is such evolution of this cryptic variation in *A. bipunctata* in nature? It is unlikely to evolve in the wingless phenotype of *A. bipunctata*, since it is rare in the wild and no long-term adaptive value of winglessness seems very likely in this species. Thus, wingless individuals show reduced fitness traits related to development time, life span, and reproduction compared to wild-type beetles (Ueno et al. 2004), and wingless females are less frequently mated with in the laboratory (S. T. E. Lommen, E. V. Bitume, P. W. de Jong, P. M. Brakefield, unpublished data). However, cryptic variation may evolve in a wild type phenotype when this variation has pleiotropic effects on fitness traits (Duveau and Felix 2012). This could be examined in our system by introgressing the winged phenotype into the wingless lines artificially selected for low and high degree of winglessness.

Evolvability of wing reduction and evolution of wingless insect morphs

The evolvability demonstrated in our artificial selection experiment is of interest in the context of the evolution of wingless morphs in other insects. In species where winglessness is an adaptive trait in nature, the wingless phenotypes are typically monomorphic and typically lack any wing tissue. One model for the evolution of such extreme wingless phenotypes is a gradual reduction of wing tissue over time as has been suggested for carabid ground beetles (Den Boer et al. 1980), wingless populations of Hawaiian brown lace wings (Tauber et al. 2007), and the wingless castes of ants (Nahmad et al. 2008). Our study shows there are no developmental constraints to the production of a continuous phenotypic range in the degree of wing reduction in a laboratory stock of *A. bipunctata*, and that intermediate phenotypes can be readily produced in this species. We have

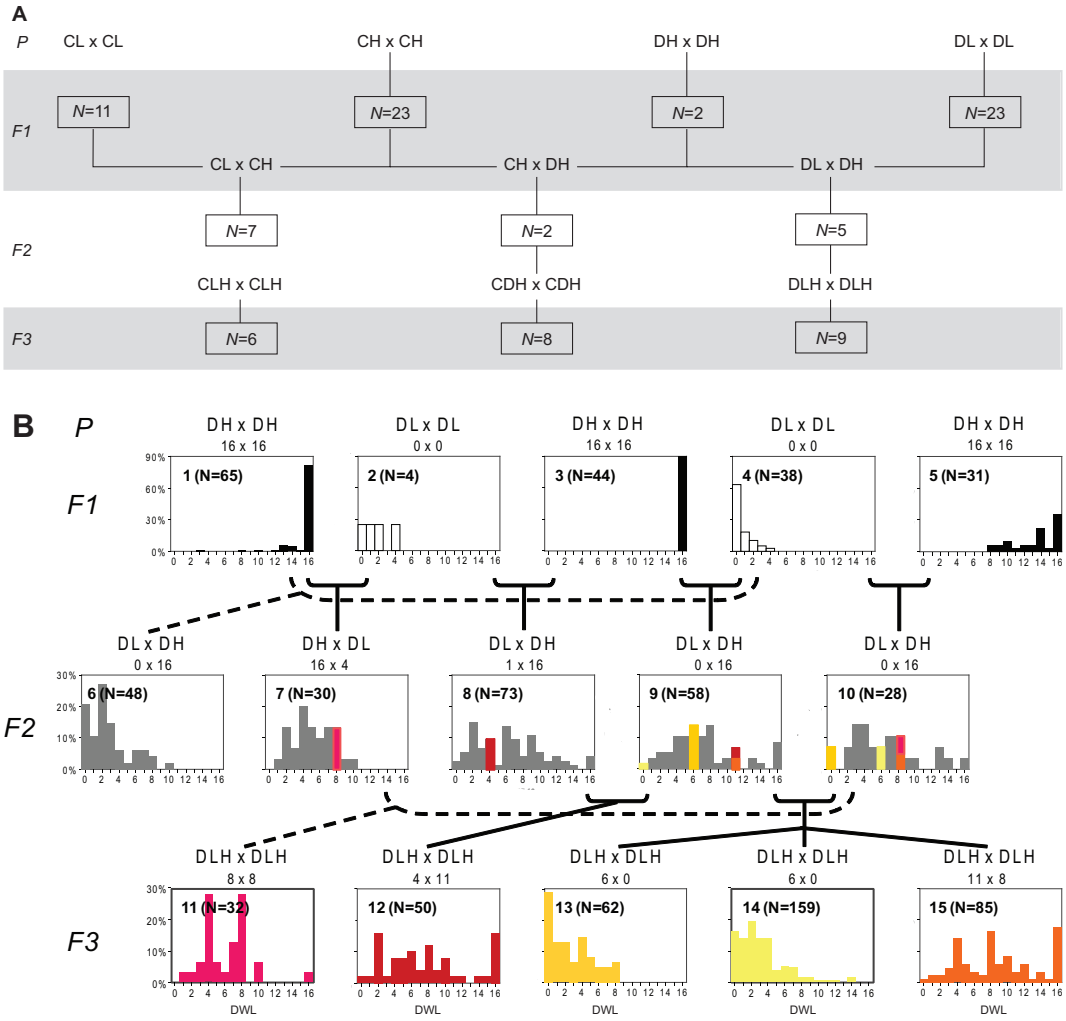


Figure 5. Pedigrees established from beetles artificially selected for extreme degrees of winglessness. (A) Scheme of the entire pedigree set-up, comprising generations *P*, *F1*, *F2*, and *F3*. C and D are replicate lines from the artificial selection experiment, and L and H indicate downward and upward directions of selection (altogether giving four selection lines: CL, CH, DL, and DH). *N* is the number of families bred; only 1-4 of these were used as sources for the next generation. (B) A part of the pedigree constituting fifteen families descending from line DL and DH. Histograms are numbered 1-15, and show frequency distributions of the degree of winglessness (DWL) in percentage values with family sizes (*N*) in brackets. The first line above each histogram indicates the type of cross corresponding to the scheme in Figure part A, whereas the second line gives male (left) and female (right) parent DWL. Note that the scale of the y-axis of the *F1* families differs from that of the subsequent generations. *F1* families from DL and DH are indicated in white and black, respectively. *F2* families (all resulting from a DL x DH cross) are presented in grey, with the categories of those individuals selected as parents of the *F3*, each marked in the colour of the corresponding *F3* family they produced. The pedigree suggests that at least two polymorphic modifier genes regulate DWL.

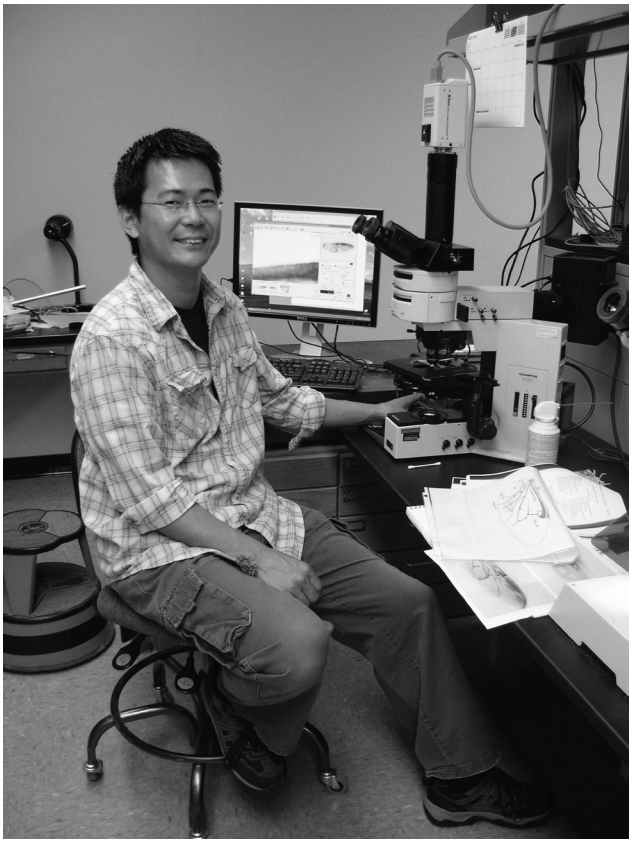
demonstrated that such cryptic variation can include a heritable component that natural selection could act upon. Therefore, even though winglessness is unlikely to be adaptive in *A. bipunctata*, our study illustrates the potential for gradual evolution. The cryptic variation released within the 'wingless' morph in our study could mimic a transitory evolutionary phase from an ancestral monomorphic winged population towards a dimorphic population with discrete phenotypes, or alternatively a monomorphic wingless species.

Evolvability of wing reduction in wingless A. bipunctata and use in biological control

Finally, the evolvability of the degree of wing reduction in *A. bipunctata* could be exploited for their application in biological control of aphids in crops or in trees in urban environments. The flight dispersal of adults is considered to reduce ladybirds' effectiveness in commercial releases (Kieckhefer and Olson 1974; Ignoffo et al. 1977; Obrycki and Kring 1998). Flightless morphs have been shown to exhibit longer residence times, and sometimes to yield better control of aphids compared to their conspecifics capable of flight (Lommen et al. 2008; Seko et al. 2008). The major obstacle for the commercialization of wingless *A. bipunctata*, however, is their reduced fitness (Ueno et al. 2004) which complicates mass breeding. Earlier work indicated that the extent of wing reduction in wingless *A. bipunctata* is negatively correlated to components of fitness (Ueno et al. 2004). Our artificial selection experiment corroborates this, because the number of offspring produced by the lines selected in the upward direction is higher than that in the corresponding line selected downward. The ability to artificially select for less wing reduction, in combination with a specific rearing temperature, as shown in this paper, could thus provide a very practical way of improving fitness. This method could ease commercial-scale breeding and, provided their flightless behaviour is retained, may also enhance the wingless ladybird's effectiveness as a biological control agent. This would provide an example of how evolutionary genetic principles can be exploited to improve pest management (Gould 2008).

Acknowledgements

We are grateful to the late Hideki Ueno for his inspiring ideas regarding the genetics of winglessness in *A. bipunctata*, and to Ayako Ueno who found the homozygous wingless individual that was used to found the laboratory population. We thank Patrícia Beldade and Fons Debets for discussion, Cerise Allen and Agnieszka Doroszuk for valuable comments on earlier versions of the manuscript, and Nicolien Pul for help with the figures. Koppert B.V. kindly provided *Ephestia* eggs, and Olympus provided photography equipment.



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