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# PART B

Causes and consequences of variation  
in wing length in *Adalia bipunctata*



A melanic, wingless male mating a typical, winged female

# 4

## Genetic linkage between melanism and winglessness in the ladybird beetle *Adalia bipunctata*

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## Abstract

We report a case of genetic linkage between the two major loci underlying different wing traits in the two-spot ladybird beetle, *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae): melanism and winglessness. The loci are estimated to be 38.8 cM apart on one of the nine autosomes. This linkage is likely to facilitate the unravelling of the genetics of these traits. These traits are of interest in the context of the evolution of intraspecific morphological diversity, and for the application of ladybird beetles in biological control programs.

### **Keywords**

*Adalia bipunctata*, classical genetics, linkage disequilibrium, wing morphology

## Introduction

Variation in insect wing morphology has long been studied from different perspectives. Examples include both fundamental studies in the context of the evolution and development of phenotypic variation, and applied studies in the context of pest control. The genetics underlying such variation can help to better understand the nature of the variation in either of the contexts. We report here genetic linkage of two morphological wing traits, melanism and winglessness, in the ladybird beetle, *Adalia bipunctata* (L.).

### Melanism in ladybirds

Intraspecific variation in colour patterns of ladybird beetles (Coleoptera: Coccinellidae) has been studied for many decades (Dobzhansky 1924, 1933; Lusia 1961; Majerus 1994; Majerus 1998). Generally, the elytra (the pair of forewings that are sclerotized in beetles) of ladybird beetles vary in the localization and extent of melanin, a dark pigment (Majerus 1998). In most species, there is a strong hereditary component determining melanism. There is temporal and geographic variation in frequencies of melanic individuals (Timofeeff-Ressovsky 1940; Creed 1975; Bengtson and Hagen 1977; Majerus and Zakharov 2000; Brakefield and de Jong 2011; Michie et al. 2011), and there is an ongoing debate about the nature of the selective forces maintaining colour polymorphisms in different species. Proposed theories include those related to thermal properties of black elytra ('thermal melanism') (Lusia 1961; Brakefield 1984c; Brakefield and Willmer 1985; De Jong et al. 1996; Michie et al. 2010), mimicry (Brakefield 1985), and mating preferences (Majerus et al. 1986). In the past two decades, the genetics underlying variation in melanism have been unravelled for several species of insects. Studies of *Drosophila* spp. have helped to decipher the melanin biosynthesis pathway, and the effector genes involved in this canonical cascade seem well conserved throughout the insects (True 2003; reviewed by Wittkopp and Beldade 2009; Van 't Hof and Saccheri 2010). The mechanisms responsible for regulation of the positioning of melanin in space and time, are however, far more diverse (Wittkopp et al. 2003; Van 't Hof et al. 2011), and have not been elucidated for ladybird beetles.

### Winglessness in ladybirds

Some species of ladybird beetles also exhibit natural variation in wing length (Pope 1977; Hammond 1985; Majerus 1994). Little is known about the development and evolution of this trait (Pope 1977; Lommen et al. 2009), but interest has grown in the potential use of wingless morphs in biological control programs involving ladybirds as natural enemies (Lommen et al. 2008; Obrycki et al. 2009).

### **Melanism and winglessness in *A. bipunctata***

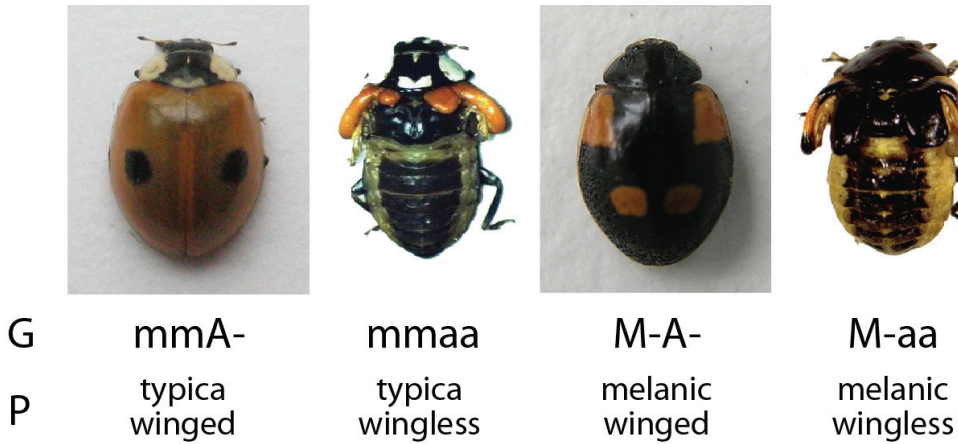
In some natural populations of the ladybird, *A. bipunctata*, both elytral colour pattern and elytron length are polymorphic (Lusis 1961; Marples et al. 1993) (Fig. 1). With respect to the colour polymorphism, hundreds of different colour morphs have been described, but three predominate in nature: the typical, non-melanic morph which has red elytra with two black spots (*typica*), and the melanic morphs with black elytra and with either four (*quadrimaculata*) or six (*sexpustulata*) red spots (Brakefield 1984a; Majerus 1998). It has been proposed that variation in elytra colouring is determined by a series of alleles at a major supergene, hierarchical in dominance, with melanic morphs generally dominant over non-melanics (Majerus 1998). However, the location of this supergene in the genome, and the sequence of the alternative alleles at this locus, are still unknown.

Typically, elytra cover the entire abdomen and the folded flight wings of *A. bipunctata*. In contrast, in wingless morphs, both pairs of wings (elytra and flight wings) are truncated, such that the abdominal part of the abdomen is not entirely covered by the elytra (Lommen et al. 2009). Wingless morphs are rare, but have been recorded for at least one population in The Netherlands (Marples et al. 1993), and five in the United Kingdom (M.E.N. Majerus, personal communication). So far, no fitness advantages have been found for the wingless morphs. The wild type is favoured for several life history traits (Ueno et al. 2004), and wild type females are more frequently mated by males than wingless ones (this thesis, chapter 7). Therefore, the potential adaptive value of winglessness in *A. bipunctata* in nature, if any, is unknown. Nevertheless, wingless *A. bipunctata* have been shown to be successful in biological control of aphids (Lommen et al. 2008; 2012). Winglessness (the presence of the truncation) is determined by one major, single locus with the winged, wild type allele being dominant over the wingless one (Marples et al. 1993). The location of the locus and the sequence of its alternative alleles, remain obscure. In this paper, we examine the degree of genetic linkage between these two wing traits, elytral melanism and winglessness, in *A. bipunctata* in classical genetic crosses.

### **The study population**

Our observations on linkage between the wingless trait and elytral colour were all made in a laboratory population of wingless *A. bipunctata*. The origin of this population is described in detail by (2005). In summary, the ancestors of the population were all collected in the Utrecht region in The Netherlands, and one of them was wingless (homozygote recessive for the wingless trait). The frequency of the wingless alleles in the laboratory population was elevated to 100% by selecting wingless phenotypes in the F2 generation and breeding them together. To maintain a diverse genetic background, they were outcrossed to wild type beetles every now and then.





**Figure 1.** Variation in elytral colour and winglessness in *Adalia bipunctata*. Below each photograph, the corresponding phenotypes (P) and genotypes (G) are given, with letters representing the alternative alleles at both loci (M = melanic allele, m = typical allele, A = winged allele, a = wingless allele), with capital letters dominant over lower case letters.

**Table 1.** Linkage between the genes for elytral colour and winglessness in a laboratory population of *A. bipunctata*. Data are shown in separate rows for one group cross and for each of eleven individual families (fam). For the four largest families, the results are additionally split for sons and daughters. For each cross, the first grey block gives the parental genotypes, followed by the distribution of genotypes in the F1 offspring in the white block, with letters corresponding to the alleles (M = melanic allele, m = typical allele, A = winged allele, a = wingless allele), and capital letters dominant over lower case letters. The second grey block gives details on the linkage. Recombination fractions and map distances are only given for families with at least 50 offspring. G = value of G following a G-test of independence; df = degrees of freedom; P = probability value resulting from the G-test (significant p-values are indicated by an asterisk); RF = fraction of recombinant genotypes of the total offspring number; map distance according to Haldane's map function (in centiMorgans).

cross	parental genotypes		F1 offspring numbers per genotype (observed (expected))					genetic linkage				
	♂	♀	total	MmA <sup>a</sup>	mmaa <sup>a</sup>	mMaa <sup>b</sup>	Mmaa <sup>b</sup>	G	df	P	RF	map distance (cM)
group	MmAa	mmaa	2426	1044 (778)	722 (456)	365 (631)	295 (561)	499.96	1	0.000*	0.27	38.8
fam 7	mmaa	MmAa	376	138 (102)	122 (86)	61 (97)	55 (91)	56.32	1	0.000*	0.31	48.0
		♂	191	75 (57)	57 (39)	31 (49)	28 (46)	27.74	1	0.000*		
		♀	185	63 (45)	65 (47)	30 (48)	27 (45)	28.01	1	0.000*		
fam 9	mmaa	MmAa	358	132 (99)	112 (79)	46 (79)	68 (101)	49.27	1	0.000*	0.32	50.6
		♂	167	62 (47)	51 (36)	20 (35)	34 (49)	22.23	1	0.000*		
		♀	191	70 (52)	61 (43)	26 (44)	34 (52)	27.21	1	0.000*		
fam 16	mmaa	MmAa	172	75 (52)	58 (35)	21 (44)	18 (41)	53.23	1	0.000*	0.23	30.2
		♂	100	38 (23)	42 (27)	9 (24)	11 (26)	38.55	1	0.000*		
		♀	72	37 (30)	16 (9)	12 (19)	7 (14)	13.41	1	0.000*		
fam 18	mmaa	MmAa	36	15 (10)	12 (7)	7 (12)	2 (7)	10.79	1	0.001*		
fam 25	mmaa	MmAa	82	26 (16)	35 (25)	7 (17)	14 (24)	20.89	1	0.000*	0.26	35.9
fam 28	mmaa	MmAa	27	9 (8)	7 (6)	6 (7)	5 (6)	0.90	1	0.342		
fam 29	mmaa	MmAa	170	69 (46)	62 (39)	19 (42)	20 (43)	52.37	1	0.000*	0.23	30.7
		♂	87	33 (21)	34 (22)	10 (22)	10 (22)	26.79	1	0.000*		
		♀	83	36 (25)	28 (17)	9 (20)	10 (21)	25.25	1	0.000*		
fam 32	MmAa	mmaa	27	12 (8)	10 (6)	4 (8)	1 (5)	12.70	1	0.000*		
fam 46	MmAa	mmaa	45	13 (9)	17 (13)	6 (10)	9 (13)	5.12	1	0.024*		
fam 50	MmAa	mmaa	24	8 (5)	9 (6)	5 (8)	2 (5)	4.85	1	0.028*		
fam 58	MmAa	mmaa	50	25 (20)	12 (7)	7 (12)	6 (11)	9.87	1	0.002*	0.26	36.7

<sup>a</sup>parental genotype

<sup>b</sup>recombinant phenotype

## Detection of linkage between melanism and winglessness

We initially observed a correlation between melanism and winglessness while breeding the wingless population. We had composed groups of virgin, melanic, winged individuals (heterozygote for both traits) of one sex, mixed with virgin, typical, wingless individuals of the opposite sex (homozygote recessive for both traits). The F1 generation of these group crosses constituted the two parental phenotypes (typical, wingless individuals, and melanic, winged individuals), and the two recombinant phenotypes (melanic, wingless individuals, and typical, winged individuals) (Fig. 1). In total, 2426 offspring were scored for both traits. Since the wingless phenotype was less frequent than the winged one, expected frequencies of each of the four phenotypes were calculated based on allele frequencies in the total population of the F1 generation. 73% of the F1 offspring corresponded to one of the parental phenotypes, which is a significant deviation from the expected frequency, and suggesting linkage disequilibrium (Table 1). From these numbers, map distance was calculated to be 38.8 cM following Haldane's map function (Haldane 1919).

Subsequently in one of the outcrossing events, the wingless laboratory population was enriched with a further copy of a wingless allele. It originated from an individual found at the same locality that proved to be heterozygous for the wingless trait, and so all three alleles used to establish our stock were likely to be copies of the same wingless allele. We then used the enriched stock to confirm genetic linkage in similar crosses, but instead using twenty individual families. Eleven of them were bred from a melanic, winged male with a typical, wingless female, and nine from a typical, wingless male with a melanic, wingless female. All parents were virgin at the start of the experiment. Female ladybirds store sperm after mating, and lay eggs nearly daily throughout life (De Jong et al. 1998). F1 offspring were collected as long as the female laid fertilized eggs, bred to adulthood, and scored for elytral colour and winglessness. Offspring numbers varied widely from 4 to 376 with a median of 50 offspring. Because wingless *A. bipunctata* have lower longevity than winged ones (Ueno et al. 2004), the number of offspring of families with wingless mothers was lower than of those with a wingless father (Mann Whitney U-test,  $U=19.5$ ,  $P < 0.05$ ). Otherwise, there were no differences between these two types of families. Overall, nine families yielded too few offspring to allow statistical analysis and were, therefore, excluded. The results from the other eleven families are summarized in Table 1 showing offspring numbers per phenotype, the significance of genetic linkage calculated by G-tests of independences, and the estimated map distances. Ten of the eleven families showed significant linkage. A similar non-significant trend was seen in the other family. Four of the families were large enough to examine male and female offspring separately. All four demonstrated the same pattern for sons and daughters (Table 1), indicating that these loci are not on the sex chromosome. The nature of linkage was further calculated for the six families with at least 50 offspring. The recombination fraction ranged from 0.23 to 0.32 with an average of 0.27.

The map distance between the melanic and the wingless locus was estimated following Haldane's map function (Haldane 1919). It ranged from 30.2 to 50.6 cM with an average value of 38.7 cM, nearly exactly the distance initially estimated from the group crossing.

### **Conclusion & discussion: using linkage in molecular studies**

In conclusion, we have shown that the major loci for winglessness and melanism of the elytra are genetically linked on one of the nine autosomes (Smith 1953) of *A. bipunctata*. Knowledge of this linkage may facilitate future molecular studies on the genetic bases of these two traits in *A. bipunctata*, even though the loci are not closely linked. A candidate gene approach can attempt to identify these two loci in *A. bipunctata*. Developmental studies on *A. bipunctata* have suggested that genes involved in dorso-ventral wing patterning provide candidates for winglessness (Lommen et al. 2009). Genetic tools, such as germ line transformation and RNA interference, are being developed for ladybirds (Kuwayama et al. 2006), and may further speed up such investigations.

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The rearing chamber with beetles in plastic dishes