The handle http://hdl.handle.net/1887/20872 holds various files of this Leiden University dissertation.

**Author:** Lommen, Suzanne Theresia Esther  
**Title:** Exploring and exploiting natural variation in the wings of a predatory ladybird beetle for biological control  
**Issue Date:** 2013-05-16
PART C

Synthesis and outlook
The logo of the Evolutionary Biology Group (a compilation of our study insects, made by Nicolien Pul), the group in 2007, and my office
Synthesis

Suzanne T. E. Lommen
In this thesis, I explore natural variation in wing length of the two-spot ladybird beetle, *Adalia bipunctata*, and exploit this knowledge to improve the effectiveness of this natural enemy for the biological control of aphids. I take a multidisciplinary, experimental approach to examine the causes and consequences of wing length variation in this species. The thesis includes genetical, developmental, behavioural, life-history, and population-dynamic studies. I discuss these results, on the one hand in an evolutionary perspective, and on the other, in the context of biological control.

In this chapter, I first provide a short background to the study system, the evolution of flightlessness, and ladybirds as biological control agents. I then summarize the experimental work presented in the previous chapters. Next, I integrate the results of this work to address the evolution of the wingless character in *A. bipunctata*. Following this, I discuss the potential for wingless morphs of *A. bipunctata* to improve the biological control of aphids compared to winged conspecifics, and propose how variation in wing length morphology might be further exploited to enhance the use of wingless beetles as biological control agents.

**Background**

*Adalia bipunctata* is a predatory ladybird beetle native to large parts of Europe and North America. This generalist species feeds on a wide range of aphid species. “Wingless” adult morphs of *A. bipunctata* occur naturally in at least one population in The Netherlands (Marples et al. 1993) and five in the United Kingdom (Majerus and Kearns 1989, M. E. N. Majerus personal communication), but they are rare. In the wingless morph both pairs of wings are truncated, and this malformation is regulated by a recessive allele at a single locus (Marples et al. 1993; Ueno et al. 2004). The frequency of the wingless allele in the field, is however, unknown. In laboratory populations fixed for this wingless trait, cryptic variation for the extent of wing reduction was revealed (Marples et al. 1993; Ueno et al. 2004). There is a continuous range of wingless phenotypes, from individuals lacking all wing tissue to those only missing a small piece of the tip, but the underlying mechanisms regulating this variation is unknown.

Flightlessness occurs in many species of insects, and flightless morphs typically have reduced or no wings. In some insect species, the evolution of flightlessness is considered to be adaptive, and thus to result from natural selection in its favour. This has often been explained by a trade-off between the ability to disperse by flight, and reproduction (Wagner and Liebherr 1992). However, among ladybirds (Coccinellidae), winglessness is uncommon (Pope 1977). In this thesis, I ask why winglessness, and variation in the degree of wing reduction, exists in *A. bipunctata*. On the one hand I test the causes of wing reduction and on the other, I examine the effects of wing reduction
and potential pleiotropic effects of the loci regulating wing length.
Predatory ladybirds are used for biological control of aphids by means of inundative augmentation, where large numbers of mass-reared natural enemies are released for immediate and short-term pest control (Eilenberg et al. 2001). To be cost-effective, the effects of biological control should outweigh the production costs. Currently, the application of A. bipunctata in biological or integrated control programs is restricted, one of the reasons being that the adults tend to fly away from the target host plants. This reduces the efficacy of control, and requires repeated releases, while mass-rearing is expensive. In some other species of ladybird, limiting their ability to fly has indeed enhanced their residence time on the host plants following release, and sometimes, their efficacy in biological control of the aphids (Ignoffo et al. 1977; Ferran et al. 1998; Tourniaire et al. 1999; Weissenberger et al. 1999; Seko et al. 2008). In this thesis, I test the potential for wingless morphs of A. bipunctata to improve the biological control of aphids compared to winged morphs.

Summary of experimental work

The research described concerns experimental work using laboratory populations of A. bipunctata of which all ancestors originated from a wild, Dutch population. The experimental work is divided into two sections. Part A (chapters 2-3) concerns the efficacy of wingless A. bipunctata as biological control agent of aphids. In part B (chapters 4-7), I explore causes and consequences of natural variation in wing length in A. bipunctata.

Biological control by wingless A. bipunctata

In chapter 2 I provide the proof of principle that wingless morphs of A. bipunctata stay longer on aphid-infested plants after their release than do winged morphs, and that this can result in enhanced aphid control. However, whether or not control was improved, depended on the species of aphid used. We released a single ladybird, either winged or wingless, on single, caged pepper plants infested with either Myzus persicae or Aulocorthum solani. In control treatments, no ladybird was released. Ladybirds were monitored during 48 hours, and then the numbers of aphids remaining on the plants were counted. With both species of aphids, wingless ladybirds remained longer on the plants than winged ones. Nevertheless, both beetle morphs reduced the numbers of A. solani to a similar extent. This is likely to be explained by the tendency of A. solani to drop off the plant upon disturbance, which was probably invoked by the initial presence of a ladybird on the plant, independent of its phenotype. In contrast, numbers of M. persicae were only reduced by wingless morphs of A. bipunctata, probably because of the increased consumption as a direct effect of the prolonged residence time on the plant. In a control experiment where individual ladybirds were
fed one of these species of aphids, or a third one, on single leaves in Petri dishes, no differences in feeding behaviour were found between wingless and winged ladybirds. This indicates the wingless alleles have no pleiotropic effects on this behavioural aspect. Therefore, I conclude that wingless ladybirds can improve biological control of aphids, provided consumption of aphids is the main mode of aphid population reduction.

Chapter 3 provides proof of principle that the release of wingless *A. bipunctata* can reduce aphid-borne honeydew beneath urban lime trees. Honeydew is excreted by aphids, and spoils the surface beneath the infested tree canopy. This can cause problems and risks of accidents in urban environments, and therefore, many Dutch municipalities release commercially bred *A. bipunctata*. To my knowledge, this is the first time that this potential control measure has been evaluated scientifically. We released larvae and adults of our wingless laboratory stock in two different species of lime trees (*Tilia* spp.) in a region of Amsterdam after natural infestation by the lime aphid, *Eucallipterus tiliae* L.. Releases were made in eight consecutive weeks, and during that period and one month later, the amount of honeydew dripping from these treated trees was compared to untreated ones in a randomized block design. The amount of honeydew was measured by exposing water-sensitive papers beneath the canopy in a standardized way. The amount of honeydew was approximately halved in treated trees compared to controls. Because both larvae and adults of the wingless strain were released, it remains unclear whether the observed effects can be attributed to the flightless characteristic of this strain. It is also questionable whether this reduction is satisfactory for reducing the associated nuisance to the city population. Finally the applied method should be modified to become cost-effective.

**Genetics of wing length variation in *A. bipunctata***

Chapter 4 shows that the genes regulating winglessness and melanization are physically linked on the same autosome. This was determined by phenotyping the offspring from families of parents varying in these characteristics of the elytra. This genetic linkage might facilitate the identification of loci regulating these traits.

In chapter 5, I develop an extended model for the genetic architecture of winglessness in *A. bipunctata*. The most parsimonious model contains three polymorphic autosomal loci, of which a single bi-allelic gene regulates the wing status with the winged allele dominant to the wingless one (Marples et al. 1993; Ueno et al. 2004), and two other polymorphic genes interact with the environment to regulate the expression of the wingless trait (chapter 5). The model for the expression of the trait resulted from a series of studies using methods of classical genetics. First, we revealed a strong family-by-temperature interaction on the variation in the extent of wing reduction by rearing offspring of 41 wingless families at two different temperatures. Heritability of the degree of wing reduction was 0.64±0.09 at 19°C and 0.29±0.06 at 29°C. Second, artificial selection on the degree
of wing reduction at 20°C over five generations demonstrated that the degree of wing reduction can be altered rapidly. Both extreme phenotypes (individuals without any wing tissue versus individuals resembling winged wild types) were obtained within a few generations by selection in opposite directions in two replicates. It was also noticed that the upward selection lines had a higher reproduction than the corresponding downward selected lines. Third, a pedigree analysis of 96 wingless families derived from these selection lines covering four generations indicated that the heritable component of the variation in wing reduction encompasses at least two polymorphic genes. The heritable variation in the degree of wing reduction observed in wingless morphs is cryptic in winged morphs, but has its origins in standing genetic variation in the wild population.

I reason that this phenotypic variation in wingless *A. bipunctata* corresponds to an evolutionary model of gradual wing loss, which predicts that winged species evolve to become wingless by gradual loss of wing tissue over time. However, I recognize that such evolution is not likely to occur in *A. bipunctata* since evidence for the adaptive value of the wingless state is lacking. Nevertheless, our study illustrates the potential of developmental mechanisms for gradual evolution. Finally, I argue that the manipulation of wing length, as achieved in the artificial selection experiment, can be exploited to improve biological pest control by wingless ladybird beetles.

**Development of wing length variation in *A. bipunctata***

In Chapter 6, I conclude that the development of the wings is delayed in the larval stage of wingless morphs resulting in incomplete wings at the time of pupation, and consequently, yielding adults with truncated wings. This was discovered by a series of morphological observations and measurements. Larval stages were examined histologically. Because wings begin to develop underneath the skin in these stages, the method was by necessity destructive. Therefore, wingless families that differed in their degree of wing reduction were reared, and their offspring compared to individuals from the wild type stock. After pupation, the outline of the wings is visible externally from the outside, and therefore, the shape and size of wings could be compared within individuals both in the pupal stage and later in the eclosed adult. Morphometric measurements on adults showed that the degree of truncation is closely correlated between the two pairs of wings (elytra and the flight wings), suggesting a coupled developmental mechanism. However, the posterior part of the wing was, on average, reduced to a larger extent than the anterior part. Finally, gene expression studies in larval wing tissue using immunohistochemistry of some important candidate wing development genes known from *Drosophila* (*nubbin*, *engrailed*, and *Distal-less*), showed that the expression of *Distal-less* was incomplete in larvae of wingless beetles. This pattern generally corresponded to the truncation patterns observed in adult wing phenotypes: this gene is typically expressed along the entire wing margin, but expression was lacking in the distal tip, extending to a variable extent towards the posterior, and sometimes the anterior margin. Since *Distal-less* is part of the
dorso-ventral patterning gene cascade, I suggest that modifications in this patterning pathway may explain both the incomplete expression of this gene and the truncation in wings of adult wingless *A. bipunctata*. However, it is not understood how such developmental flaw is connected to the asynchronic development of wing tissue, and how this would yield truncated wings.

**The role of elytra in mating, and consequences for wingless *A. bipunctata***

In Chapter 7, I argue that the female elytral structure plays a role in mating in *A. bipunctata* by means of tactile cues, and that therefore, the probability wingless females mating declines with decreasing length of their elytra. Mating behaviour experiments were performed in small Petri dishes where individual males were offered either one or two females (no-choice and choice experiments, respectively). Their behaviour was then recorded for fifteen minutes. Four types of ladybirds were used: phenotypically wingless individuals (homozygous recessive for the major wingless gene), and phenotypically winged individuals with either no (wild-type), one (heterozygous) or two wingless alleles (genetically wingless, but artificially selected for long elytra). Comparing the behaviour of these types provided no evidence for pleiotropic effects of the wingless allele on mating behaviour. Male-female interactions were strongly affected by the female phenotype, but not the male phenotype. The incidence of mating decreased with female elytra length in no-choice tests, and in choice tests with a winged and a wingless female, the latter were never mated. These studies were limited to a very short time frame, and I expect that the frequency of mating with wingless females will increase if males have no choice but are given more time. Thus, I am not concerned that winglessness will hamper mating in mass-rearing. In contrast, it is hard to predict the mating success of wingless individuals in the field, since flight can be important for males in finding mates, but may also be a means for females to escape mating.

**Evolution of winglessness in *Adalia bipunctata***

I have examined causes and consequences of variation in wing length in *A. bipunctata* (part B). I will now use the results to discuss whether natural selection can explain the evolution of winglessness in the Utrecht population of *A. bipunctata*.

**Causes of wing length reduction**

I have discovered several mechanisms related to the development of reduced wings:

- The major locus regulating winglessness is linked to melanism (chapter 4).
- In addition to the major locus regulating winglessness (Ueno et al. 2004), at least two other polymorphic loci regulate the expression of this trait in our stock, as does environmental
temperature (chapter 5).

- In wingless morphs, the development of the wing tissue in the larval stages L3 and L4 is delayed, and as a result, the wing tissue is incomplete at the time of pupation, which then results in adults with truncated wings after metamorphosis (chapter 6). Within the wingless phenotype, the rate of larval wing development seems to be positively correlated to the length of the adult elytra (chapter 6).
- The wingless genotype is associated with a lack of expression of the gene Distal-less in the larval wing tissue (chapter 6).
- The wingless allele has no effect on the development of flight muscles (S. V. Saenko and S. T. E. Lommen, unpublished results).

A next step towards fully understanding the mechanistic causes of variation in wing length, would be to link the underlying genetics to the developmental mechanisms. In other words, to identify the loci regulating winglessness and variation in the extent of wing reduction, and to find out how these loci are associated with the development of the larval wing tissue. One approach could be to use the deviations in wing development to select candidate genes, and to consequently test their function in A. bipunctata by establishing RNA interference in the larvae, and phenotyping the emerging adults (Tomoyasu and Denell 2004). Another approach could be to use families segregating for the wingless phenotype for comparative genetic analysis by sequencing.

**Consequences of the wingless allele and wing length reduction**

In addition to the causes of winglessness, I have examined some putative consequences of the wingless genotype and phenotype in A. bipunctata. Table 1 list my findings together with existing knowledge. Among all the traits investigated, wingless morphs only performed better than winged morphs with respect to pre-oviposition period and adult size. However, it is unclear if wingless females will really start to lay their eggs earlier in their lives than winged conspecifics since their development takes longer (Table 1) (Ueno et al. 2004). An increased body size can be advantageous for both females and males. In many insects, female body size is very closely positively correlated to fecundity (Honěk 1993). However, total fecundity in wingless females was much lower than that of winged females, resulting from a reduced longevity (Table 1) (Ueno et al. 2004). Perry et al. (2009) showed that larger males of A. bipunctata can more easily overcome female resistance in mating, but they did not elucidate why larger males are more successful in that. Under conditions when body size is the key factor, wingless males might thus benefit from being rejected less frequently, but when this benefit is accounted for by body weight, there should be no benefit for them since pupal weights do not differ significantly between winged and wingless beetles (Table 1).

For two traits examined here, it is not clear how they would affect fitness in the field (Table 1). First, wingless beetles, like winged ones, develop flight muscles. However, since they cannot fly,
both the development of these muscles and their use in moving truncated wings (K.G. Koops & S. T. E. Lommen, unpublished results) seems a waste of energy. Muscles are costly to develop since they are rich in proteins. In some species this has been shown to come at the cost of reproduction. For example, in female *Gryllus firmus* crickets, the mass of the dorsolongitudinal flight muscles (the main flight muscles) is negatively correlated with ovary mass in females (Roff et al. 2002). It is hypothesized that in the evolution of wingless morphs, dispersal was first inhibited prior to a decrease in flight muscle development, and before the wings were finally reduced (Roff 1986).

Examination of some wingless insects indeed confirms the (partial) loss of flight muscles (e.g. Jackson 1928; Smith 1964; Zera and Mole 1994). Second, female mating frequency of wingless females was lower than that of winged females in 15-minute long trials, but it is unlikely that these results can be translated directly to a field situation where individuals are not limited in space and time. Moreover, mating involves both benefits (reproduction), and costs (superfluous matings are costly (Perry et al. 2009), and mating involves the risk of sexually-transmitted diseases (Hurst et al. 1995)). Thus it is hard to predict how these results affect natural selection.

Finally, the fitness of wingless morphs is reduced compared to that of wildtypes with respect to the six remaining life-history traits, and flight ability (Table 1). In addition, removal of the elytra resulted in a higher predation rate of the ladybird beetle *Coccinella septempunctata* L. by a bird, which was explained by the aposematic function of the elytral colour pattern (Dolenska et al. 2009).

**The evolution of winglessness in *Adalia bipunctata***

Overall, wingless phenotypes of *A. bipunctata* show a reduction in many important fitness traits, and this disadvantage probably largely outweighs any fitness benefits resulting from the few traits where they perform better. Sometimes, the existence of traits that are disadvantageous in homozygotes, can be explained by increased fitness of the heterozygotes. However, Ueno et al. (2004) suggest a negative pleiotropic effect of the wingless allele on the fitness of *A. bipuncta* heterozygous for the wingless trait. In conclusion, these results indicate the wingless allele will be selected against by natural selection (Ueno et al. 2004).

This is not surprising, given that this wingless genotype in *A. bipunctata* corresponds to a reduction in both pairs of wings, and not only flight wings. In other flightless beetles typically only the flight wings have been reduced (Darlington 1936; Smith 1964; Dybas 1978; Roff 1990), whereas the elytra are normally not changed in size (but see Dybas 1978), only sometimes have become fused or rigidly locked (Darlington 1936; Smith 1964). Elytra fulfill an important role in the protection of ladybirds against predators and adverse environments (e.g. they protect against dessiccation at low humidity) (Crowson 1981), and play a role in mating behaviour. Indeed, in wingless *A. bipunctata*, lacking both flight wings and elytra, not only flight is impaired, but also many fitness traits and mating behaviour are affected (Table 1). Thus, generally, there seems to be a strong constraint on
the loss of elytral tissue. In addition, wingless *A. bipunctata* exhibit fully developed flight muscles, which are costly to develop but do not even contribute to an increased dispersal.

The wingless allele in the Utrecht population has presumably been maintained by other evolutionary processes: a balance between recurrent mutation (explaining the origin of the wingless allele) and selection (negative natural selection explaining the disappearance of the wingless allele), or by genetic drift. Mutation hotspots are commonly observed in genomic sequences and may explain recurrent mutations (Maki 2002; Tian et al. 2008). A recent mutation-accumulation study in *Caenorhabditis elegans* Maupas shows that some phenotypic variants are much more often induced by mutation accumulation than could be expected, and this was explained by developmental processes (Braendle et al. 2012). Genetic drift is a stochastic processes, and is more likely to occur in small populations (Gillespie 2006).

To evaluate the likelihood of these alternative evolutionary processes as an explanation for the existence of the wingless allele in *A. bipunctata*, population genetic studies should be conducted among several populations, preferrably including others where winglessness was found. These can estimate the frequency of the wingless allele and the size of these populations, which can then be used to test predictions related to the different processes. At present, the evolution of winglessness in *A. bipunctata* remains puzzling.

**Table 1.** The performance of wingless beetles compared to winged morphs with respect to several important fitness traits. For each trait, the second column indicates how wingless phenotypes perform compared to winged ones, and the third column indicates the prediction whether that would result in lower (-), equal (=), or higher (+) fitness in the field.

<table>
<thead>
<tr>
<th>trait</th>
<th>performance of WL compared to W</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>larval development time</td>
<td>longer</td>
<td>Ueno et. al 2004</td>
</tr>
<tr>
<td>pupal development time</td>
<td>longer</td>
<td>Ueno et. al 2004</td>
</tr>
<tr>
<td>juvenile mortality</td>
<td>higher</td>
<td>Ueno et. al 2004</td>
</tr>
<tr>
<td>pupal weight</td>
<td>equal</td>
<td>Ueno et. al 2004</td>
</tr>
<tr>
<td>adult size</td>
<td>larger</td>
<td>+ M. E. van Schoor, S. T. E. Lommen, P. W. De Jong &amp; P. M. Brakefield, unpublished results</td>
</tr>
<tr>
<td>development of flight muscles</td>
<td>equal</td>
<td>? S.V. Saenko &amp; S. T. E. Lommen, unpublished results</td>
</tr>
<tr>
<td>flight ability</td>
<td>lower (typically not at all)</td>
<td>- S. T. E. Lommen, K. G. Koops, P. W. De Jong &amp; P. M. Brakefield, unpublished results</td>
</tr>
<tr>
<td>pre-oviposition period</td>
<td>shorter</td>
<td>+ Ueno et. al 2004</td>
</tr>
<tr>
<td>oviposition period</td>
<td>shorter</td>
<td>- Ueno et. al 2004</td>
</tr>
<tr>
<td>adult longevity</td>
<td>shorter</td>
<td>- Ueno et. al 2004</td>
</tr>
<tr>
<td>female aphid consumption behaviour</td>
<td>equal</td>
<td>= chapter 3</td>
</tr>
<tr>
<td>female mating frequency</td>
<td>lower</td>
<td>? chapter 7</td>
</tr>
<tr>
<td>male mating frequency/behaviour</td>
<td>equal</td>
<td>= chapter 7</td>
</tr>
<tr>
<td>total female fecundity</td>
<td>lower</td>
<td>- Ueno et. al 2004</td>
</tr>
</tbody>
</table>
The potential of wingless *A. bipunctata* in biological control of aphids

**Efficacy in aphid control**

In this thesis, I examine the effect of the release of a wingless strain of *A. bipunctata* on aphid control (Part A). For the first time, I show that naturally wingless morphs of *A. bipunctata* have longer residence times than winged conspecifics after release on to aphid-infested plants, which can consequently result in better control (chapter 2). I also demonstrate for the first time that release of this wingless strain greatly reduces aphid-borne honeydew beneath urban lime trees (chapter 3). These results add to the growing body of evidence that inundation of predatory ladybirds with impaired flight ability can help to control aphid pests. I argue that the use of natural wingless morphs of *A. bipunctata* may be preferred over the use of exotic species in areas native to *A. bipunctata*.

We have found no pleiotropic effects of the wingless allele on female consumption behaviour (chapter 2) or male mating behaviour (chapter 7). Therefore, wingless beetles seem to behave in a similar way to wild types, and the comprehensive knowledge about behaviour of *A. bipunctata* (Wratten 1976; Hemptinne et al. 1996; Omkar and Pervez 2005; Hodek et al. 2012) can probably also be applied to wingless morphs.

To further assess the potential for wingless *A. bipunctata* in biological control, their efficacy should be investigated in more detail in relation to the timing and frequency of release, the numbers and stages released, and the way they are distributed in the crop. Their dispersal range is likely to be smaller than that of conspecifics capable of flight, which has implications for the location and the number of points of release in the crop. However, wingless adults can disperse effectively in the crop by walking (Tourniaire et al. 1999, S.T.E. Lommen and K. G. Koops, unpublished results). It is unlikely that releasing them is more costly than in current practice, where larvae are released in hotspots of aphids (J. van Schelt, Koppert B.V., personal communication).

Such experiments to refine the application should be carried out on a large scale. To examine whether the release of flightless beetles will result in reproduction in the crop, and whether another generation can consequently prevent the establishment of new aphid infestations, more detailed behavioural and long-term population dynamics studies should be conducted after release. In order to assess the potential benefit of wingless beetles over wild types, future studies should include comparisons with the release of winged beetles from the same source population and a similar history in the laboratory. Finally, the interactions between the plant species, aphid species and the ladybird (e.g. aphid defence behaviour, suitability of aphid species as prey related to host plant, accessibility of host plant for the ladybird) should be considered before applying wingless or winged *A. bipunctata* for biological control (Gurney and Hussey 1970; Jalali and Michaud 2012).
Costs of mass-rearing

Another requirement for the cost-effective use of wingless *A. bipunctata*, is the feasibility of cheap mass-rearing. I experienced that handling flightless ladybirds is much easier than handling those capable of flight, which saves costs of labour. However, based on previous studies on wingless *A. bipunctata*, producers of natural enemies have raised concerns regarding the reduced fitness of wingless *A. bipunctata* with respect to mass-rearing (J. van Schelt, Koppert B.V., personal communication). Indeed, Ueno et al. (2004) showed that wingless morphs have a longer development time, and a reduced life span, compared to winged conspecifics. As a consequence of their shorter lives, but a similar daily egg production (H. Ueno, unpublished results), their lifetime reproduction is also much lower. In addition, I discovered that wingless females were less likely to be mated than winged ones in fifteen-minute trials (chapter 7). However, both studies recognize that there is wide variation in the performance among wingless ladybirds with respect to these traits, and that those morphs with longer elytra generally perform better than those with shorter ones. Ueno et al. (2004) note a trend for wingless beetles with longer elytra to perform better with respect to developmental traits, and I demonstrate that the probability of being mated increases with elytron length within wingless beetles (chapter 7). I have also shown that this variation in elytron length is highly heritable, and can yield rapid responses to artificial selection (chapter 5). Therefore, I expect lines of wingless *A. bipunctata* artificially selected for increased wing length to be most suitable for mass-rearing. I show that two replicates of such selected lines indeed had a significantly higher reproduction than their corresponding lines selected in the opposite direction (chapter 5). The performance of selected lines in mass-rearing should be further verified experimentally. I have also demonstrated that the rearing temperature during juvenile stages affects the degree of wing reduction (Lommen et al. 2005; chapter 5). Therefore, choosing specific temperatures can further enhance the production of desired wingless phenotypes. The use of flightless lines with slight reductions of the wings may additionally improve aphid control further, because of an increased longevity and improved protection to desiccation. This should also be tested in biological control experiments. Selection on a morphological trait such as wing length that is correlated with life-history traits and efficacy in biological control is much easier and faster than selecting on these traits directly.

Generally, the rearing of ladybirds is considered costly because of their carnivorous diet and their cannibalistic behaviour. However, advances have been made in the development of alternatives to aphids as food for commercially reared *A. bipunctata*, which do not compromise the development and reproductive performance of the beetles (De Clercq et al. 2005; Jalali et al. 2009; Bonte et al. 2010; Jalali et al. 2010). Such diets could provide a cheaper and a more stable supply of food for the species, but confer the risk of modified foraging behaviour when beetles are subsequently released on aphid-infested crops (Ettifouri and Ferran 1993). In addition, releasing the larval stage instead of adults renders less costly rearing, and is common practice for *A. bipunctata*.
Risks and solutions
If wingless strains artificially selected for long wings are going to be used for biological control, it is recommended to attempt to reduce the loss of genetic diversity. Ongoing selection for increased wing length in wingless morphs might finally restore flight ability (chapter 5). Therefore, I advise selecting for morphs that are still visually slightly distinct from wild type beetles. This would also ease segregation of phenotypes after outcrossing to wild types. Alternatively, it may be possible to uncouple the length of the elytra and the length of the flight wings by artificial antagonistic selection, and thus obtain lines with strongly truncated flight wings but slightly truncated elytra. Such antagonistic selection managed to uncouple the size of the forewings from that of the hind wings in the butterfly *Bicyclus anynana* (Butler 1879), resulting in butterflies with larger fore wings than hind wings, and vice versa (Frankino et al. 2007). Whether such modification in allometry is possible in *A. bipunctata* depends on the underlying developmental mechanisms.

The release of genetically wingless *A. bipunctata* in agricultural systems or in ornamental trees exposes them to wild populations of *A. bipunctata*. Therefore, there is a chance of mixed mating, resulting in an increased frequency of the wingless allele in natural populations. However, according to our current knowledge, wingless morphs have a disadvantage in many fitness traits, and no heterosis was found (Ueno et al. 2004). Therefore, I expect the wingless allele to be selected against by natural selection, and finally to disappear from natural populations.

Finally, since ladybird beetles are popular, the public may react adversely to the use of ‘handicapped’ specimens. The use of wingless lines artificially selected for long elytra also reduces this cosmetic problem.

Realizing a commercial wingless stock
Summarising the above, I argue that intraspecific natural variation in wing length of *A. bipunctata* can be exploited to improve the performance as biological control agent. Should extended research confirm the potential for commercialization, I suggest developing a “wingless” strain consisting of beetles with only slight reductions in wings. This process would encompass two levels of selection. First, the qualitative wingless trait should be fixed in a laboratory population of *A. bipunctata*. In principle, this only requires a single copy of the wingless allele, and two generations of rearing. Second, selection on the quantitative expression of the trait should be performed to obtain a “wingless” population with only slightly truncated wings. More generations of selection are required to fix this trait in the population (chapter 5). To prevent detrimental inbreeding effects, the numbers of individuals sampled as the initial population should be large, and the laboratory populations should be large, or outcrossed regularly to freshly sampled wild types (Wajnberg 1991; Bartlett 1993; Nunney 2003).

Since the wingless allele is recessive, and variation of wing length is cryptic in wild types (chapter 5),
the identification of the loci underlying these traits would allow developing markers to facilitate the screening of the desired genotypes in natural populations. The rapid development of molecular tools in general, and the upcoming sequencing of the genome of the ladybird *Harmonia axyridis* Pallas, would be helpful for identification, as well as the linkage between the loci regulating winglessness and melanism (chapter 4), and the recognition of some developmental processes that deviate in wingless morphs (chapter 6) may be helpful for a candidate gene approach.

**Acknowledgements**

I thank the Laboratory of Genetics and the Experimental Evolution Discussion Group of Wageningen University, as well as Jeroen van Schelt, for discussion. Peter de Jong, Paul Brakefield, and Bart Pannebakker provided valuable comments on this synthesis.
Pupa of a wingless two-spot ladybird beetle