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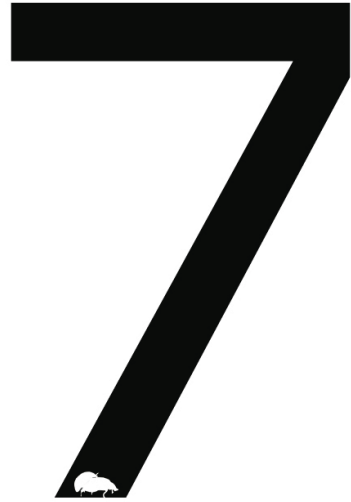


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Title: Exploring and exploiting natural variation in the wings of a predatory ladybird beetle for biological control

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Touch matters in sex: natural variation in wing length reveals tactile mating cues in a ladybird beetle

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Abstract

The elytra of many beetles play a role in mating, by providing olfactory, visual, or audible cues. In the predatory ladybird beetle, *Adalia bipunctata*, cuticular hydrocarbons covering the elytra function as contact sex pheromone. Other potential cues are not well studied in this species. Here, natural variation in the length of the elytra in *A. bipunctata* is utilized to examine their role in mating. In wingless morphs, both pairs of wings are truncated. Wingless individuals are homozygous for a recessive wingless allele, but vary in their extent of wing reduction. We recorded the behaviour of the ladybirds in laboratory mating assays where single males were offered either one or two females. Comparing the behaviour of different genotypes provided no evidence for pleiotropic effects of the wingless locus on the mating behaviour of individuals of either sex in heterozygotes or homozygotes. In contrast, mating was strongly affected by the female phenotype but not that of the male. The incidence of mating declined with female elytra length in no-choice tests, and in choice tests with single winged and wingless females, the latter were never mated. The results strongly suggest that elytra provide tactile mating cues but no visual cues in this species. We further discuss how these tactile cues could work, and then examine the implications of these findings for the evolution of winglessness and for the use of wingless ladybirds as biological control agents.

Keywords

Adalia bipunctata, behavioural observations, cuticular hydrocarbons, choice test, gas chromatography, tactile cue, visual cue, winglessness

Introduction

Cues in beetle mating behaviour

Mating behaviour of beetles (Insecta: Coleoptera) has been widely studied, especially in species of economic importance such as predators and pest species. Males are typically the most active sex in mate-searching and initiating mating behaviour, and a variety of cues for male mating behaviour have been identified. These include olfactory signals such as cuticular hydrocarbons (CHCs, “contact sex pheromones”) (e.g. in Curculionidae, Mutis et al. 2009; reviewed for Cerambycidae by Ginzl 2010; e.g. in Lampyridae, Ming and Lewis 2010) and volatile hydrocarbons (reviewed by Bartelt 2010), as well as visual (in Cerambycidae, Fukaya et al. 2005; Lelito et al. 2007; e.g. in Buprestidae, Lelito et al. 2011) tactile (e.g. in Chrysomelidae, Geiselhardt et al. 2009), and female behavioural cues (e.g. in Coccinellidae, Hemptinne et al. 1998). Experimental work is often focussed on a single type of cue, but some studies show that combinations of several cues can be involved in mating behaviour within a particular species, either simultaneously or sequentially (Hemptinne et al. 1998; Fukaya et al. 2004; Fonseca and Zarbin 2009; Yasui 2009; Lelito et al. 2011; Yasui et al. 2012).

Role of elytra in mating

Many studies cover mating cues associated with the elytra, the modified fore wings typical of beetles. Elytra usually cover the flight wings and most of the body, including part of the thorax and the entire abdomen. In many species of beetles, mating occurs with the male positioned on top of the female, thus holding her elytra. Evidently, elytra play an important role in mating, and they can provide multiple cues. For example, CHCs cover the entire body (Howard and Blomquist 1982), but given the large portion of the outer surface that is made up by this structure, and the dorsal mating position of many males, the CHCs on female elytra are the most likely body part to be contacted by a potential mating partner. Indeed, a large body of experimental work concerns male mating behaviour in response to removal of CHCs from female elytra and re-application on to both live and dead females and to artificial models (e.g. Peschke 1978; Jermy and Butt 1991; Fukaya et al. 1996; Hemptinne et al. 1998; Ginzl and Hanks 2003; Luo et al. 2011). It has been shown that CHCs are generally species-specific (e.g. Golden et al. 1992; Peterson et al. 2007; Tanigaki et al. 2007), and may be gender-specific (Ginzl et al. 2003), and condition-dependent (Peschke 1987; Steiger et al. 2007; Steiger et al. 2009). Elytra can also provide visual signals, and the role of these signals has been studied by blinding males (Pureswaran and Poland 2009) and by using rods of different colours and sizes as surrogate females (Fukaya et al. 2004; Fukaya et al. 2005). The tactile cues that elytra can provide are, however, not frequently studied.

***Adalia bipunctata* as a model in this study**

In this study, we tested the hypothesis that elytra can also play a role in mating behaviour by means of tactile cues, using the predatory two-spot ladybird beetle, *Adalia bipunctata* (L.), as a model species. Hemptinne et al. (1996; 1998) have shown that males of this species use CHCs and female-specific behaviour to recognize potential mating partners, but they found no evidence for volatile chemical cues. To investigate the putative tactile cues of the elytra, we exploited intraspecific natural variation in wing length in this species. Wingless individuals occasionally occur in the wild (Majerus and Kearns 1989; Marples et al. 1993). This trait is genetically controlled by a single locus with the wingless allele recessive to the wild-type allele (Marples et al. 1993). Genetically wingless beetles possess truncated elytra and hind wings, and the extent of wing reduction is correlated between the two pairs of wings within individuals (Lommen et al. 2009). However, the expression of the wingless trait is highly variable between individuals (Marples et al. 1993; Lommen et al. 2009). Thus, in a wingless laboratory stock of this species, phenotypes range continuously from those without any wing tissue to those indistinguishable from the wild type. This variation in the degree of wing reduction makes it a uniquely useful model organism to examine the structural function of elytra avoiding the risk of damage associated with trimming down this structure artificially. We report here on a series of mating behavioural assays comparing winged and wingless *A. bipunctata*. In control experiments we assess potential pleiotropic effects of the wingless allele on mating behaviour or mate choice. We examine the results in light of the function of elytra in mating, and then discuss observed variation in mating behaviour in relation to the evolution of winglessness and the use of this beetle as a natural enemy in biological control programs.

Material and methods

Insect rearing

All *A. bipunctata* used in the experiments came from laboratory stocks. An overview of all genotypes and phenotypes used is given in Table 1. A wingless stock (“WL”, -/- for the wingless trait) was established between 2000 and 2004 by outcrossing two individuals bearing the wingless allele to hundreds of winged beetles all collected in Utrecht in The Netherlands (details in Lommen et al. 2005). From this stock a “fully winged wingless” line (“WL_F”, -/- for the wingless trait), phenotypically close to wild types, was derived by artificially selecting for individuals with minimal wing reductions over several generations. All WL_F beetles used came from this stock. A winged stock consisting of wild types (“WT”, +/+ for the wingless trait) was established in 2005 by collecting individuals from the same Dutch locality. For the current experiment we also created a winged stock heterozygote for the wingless trait (“HET”, +/- for the wingless trait, note that the wingless allele is recessive) by

Table 1. Genotype, phenotype, description, and origin of all types of beetles tested.

type	genotype	phenotype	description	origin
wingless (WL)	-/-	wingless	variable extent of wing reduction	stock established in 2000-2004
fully-winged wingless (WL _F)	-/-	winged	artificially selected for long wings	derived from the wingless stock
heterozygous (HET)	+/-	winged	wingless gene not expressed	wingless stock crossed with wild-type stock
wild-type (WT)	+/+	winged		stock established in 2005

crossing the WL to the WT stock. All adult beetles were bred in groups separated by wing genotype, sex, and day of emergence from the pupa. They were maintained on a diet of *Ephestia kuehniella* Zeller eggs and flower pollen at a temperature of 20.5 (± 1) °C in a 16L:8D light regime.

Mating behaviour

Behavioural experiments were performed from April to August 2007 in a different climate room to that used for rearing. Because elytral colour has been found to affect female mate choice in some populations of *A. bipunctata* (Majerus et al. 1982; Majerus et al. 1986), only *typica* (red) colour morphs were used in the mating behaviour assays. All ladybirds were virgin, and since the incidence of mating is age-dependent, we standardised their age to 21 days at which most females are receptive (Hemptinne et al. 2001). We focused on male behaviour, since they spend most of their time mate-locating (Hemptinne et al. 1996) and mating is usually triggered by a male encountering a female (Hemptinne et al. 1998; S.T.E. Lommen and E.x. Bitume, personal observation). Experiments were performed in small Petri dishes (\varnothing 55 mm, 14 mm high) to exclude effects of flight ability. A single male was allowed to settle for 15 minutes before one (no-choice experiments) or two females (choice experiments) were added. We then recorded the behaviour of the male for 15 min, as well as the behaviour of the female(s) when interacting with the male. In *A. bipunctata*, mating is preceded by a fixed sequence of male behaviour initiated upon contact between the sexes (Hemptinne et al. 1998). The male first touches the female with his legs (palpate=P), then mounts her, extrudes his genitalia (attempt=A), aligns himself in the right position, and finally penetrates (copulation=C) (Fig. 1). We recorded the occurrence of these events and noted which part of the female was palpated by the male, distinguishing the front (head and pronotum) and rear of the body (remaining thorax and abdomen). We also recorded female rejection behaviour, including running, kicking, raising the abdomen, spinning, and flipping over. Although females can display rejection behaviour in all stages in the chain of sequential behaviours, it did not seem to affect male behaviour, except that it could prevent attempting males to mate or delay mating. Therefore, Figure 1 only displays rejection behaviour upon male attempt, and not earlier in the behavioural cascade.

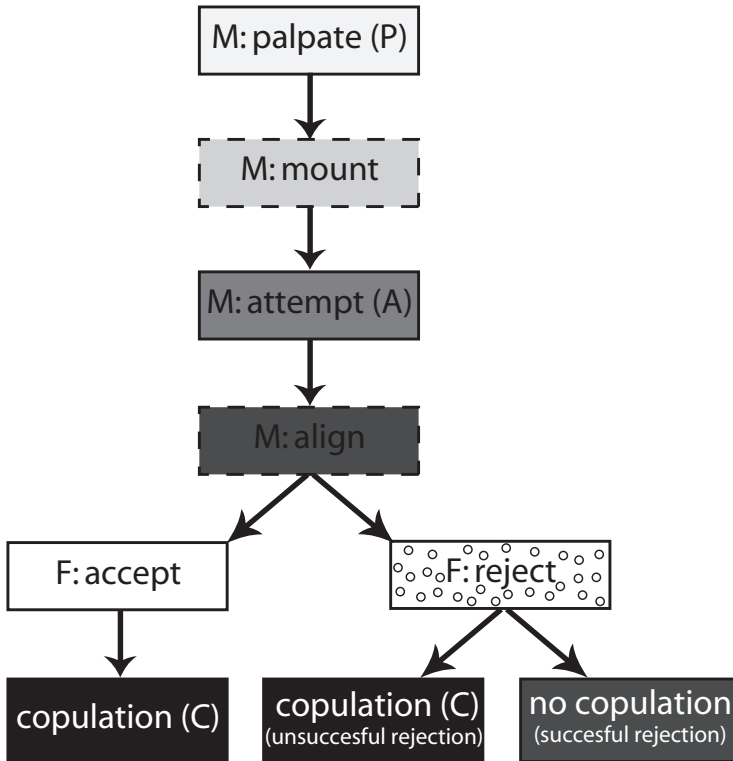


Figure 1. Sequence of mating behaviour in *A. bipunctata*. M=male, F=female behaviour. Letters in brackets indicate abbreviations used throughout the manuscript. Only behaviour in text boxes with line borders was analysed, not behaviour in boxes with dashed lines.

Individual beetles were only used once. After the trial, all ladybirds were frozen at -20°C before morphological features were assessed. Because size can affect chance of mating in *A. bipunctata* (Tomlinson et al. 1995; Yasuda and Dixon 2002; Perry et al. 2009), we measured maximum head width as an indication of body size. We determined the degree of winglessness (DWL) of all wingless beetles following Lommen et al. (2005). The categorical parameter DWL ranges from class 0 (no elytron tissue) to 5 (elytra covering more than $\frac{3}{4}$ of the abdomen). DWL classes of wingless beetles are indicated by a lower case number, thus WL_0 =wingless beetle of DWL class 0, WL_1 =wingless beetle of DWL class 1, and so on. Table 2 gives an overview of the behavioural experiments and their setup. To be able to disentangle potential pleiotropic effects of the wingless allele from direct effects, we set up a series of control experiments comparing winged phenotypes with or without wingless alleles. Subsequently we give additional details for each experiment (Table 2).

Table 2. Overview of the behavioural experiments.

experiment	objective	N	male	female
no-choice	test role of female elytra in mating	192	WT	WL, WL _f or WT
choice (1) between wingless and winged sisters	test effect of winglessness on mating behaviour and male preference	34	HET	WL vs. HET
		34	WL	WL vs. HET
choice (2) between winged females with one wingless allele or without	control experiment to test effect of a single wingless allele on mating behaviour and male preference	40	HET	WT vs. HET
		38	WT	WT vs. HET
choice (3) between winged females with two wingless alleles or without	control experiment to test effect of two wingless allele on mating behaviour and male preference	40	WT	WL _f vs. WT

No-choice experiment

To test the role of elytra in mating, single winged males (WT, N=192) were offered single females, either wild types (WT, N=30) or genetically wingless females, varying in their degree of winglessness (WL₀, N=30; WL₂, N=22; WL₃, N=29; WL₄, N=25; WL₅, N=26; WL_p, N=30).

Choice between wingless and winged sisters

We examined the effect of winglessness on male mating behaviour, including male preference and male-female interactions. By offering males a winged and a wingless female simultaneously, we assessed the effect of winglessness in females on male mating behaviour and preference. By comparing trials between winged and wingless males, we aimed to detect putative pleiotropic effects of winglessness on male behaviour. To minimise differences in the genetic background of the winged and wingless beetles, we created families with winged (HET) and wingless (WL) siblings by crossing single winged males (HET) to single wingless females (WL). A winged and a wingless brother were then each offered a pair of winged and wingless females. All four females were sisters, but were not related to the males (Fig. 1). Each family provided at most two brothers and four sisters, and the experiment was replicated 34 times.

Choice between winged females with one wingless allele or without

To identify putative pleiotropic effects of the wingless allele on mating behaviour, we compared behaviour of winged ladybirds heterozygote for the wingless trait (HET) with those wildtype for the trait (WT). We repeated the setup of the previous choice experiment (N=38 for WT males, and N=40 for HET males), but used beetles from the stocks and not from families. To distinguish WT from HET females in a trial, each of them was marked with a black dot on a different side of the pronotum; the location of the mark was alternated between genotypes over trials.

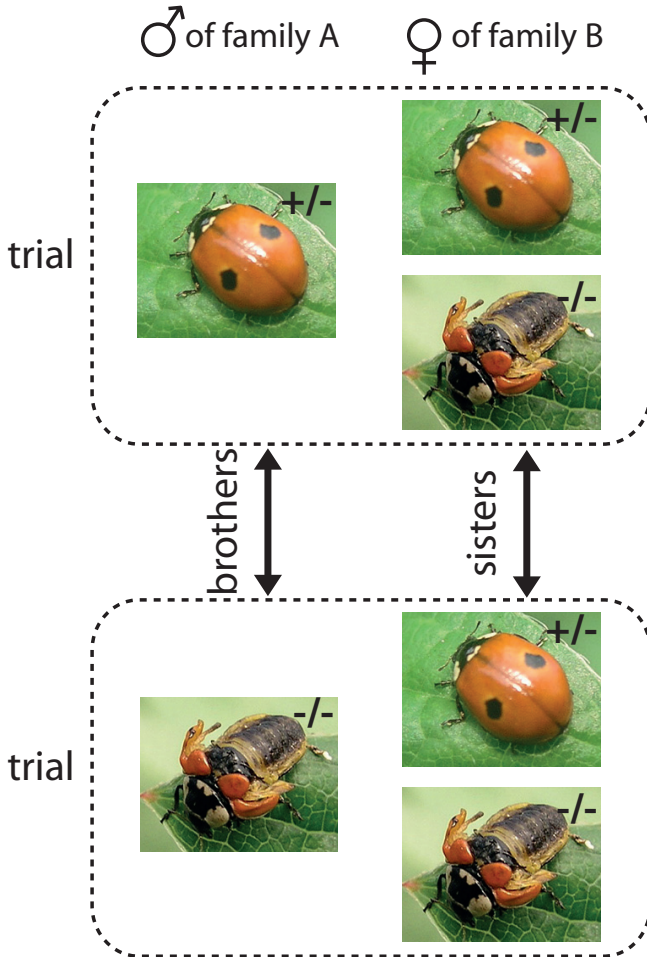


Figure 2. Setup of the mating behaviour experiment examining choice between winged (HET) and wingless siblings. Boxes are separate trials, photos indicate wing phenotypes, and wing genotype is given in the upper right corner of each photo (“+” indicating wild-type allele; “-” wingless allele). Each of the males were full sibs, and each was offered two unrelated female full sibs.

Choice between winged beetles with and without wingless alleles

To test whether female wingless alleles affect male mate choice, we offered single winged males (WT, N=40) a winged (WT) and a fully-winged wingless female (WL_f). Females were thus phenotypically similar, but were genetically different. Pairs of females were marked as above.

Statistical analyses

Data analysis used R software (version 2.6.1., R Development Core Team 2007). The few mating trials where males were so inactive that they never palpated any female were independent of male or female group, and, therefore, excluded from analysis (N=5/192 in no-choice experiment, N=2/68 in choice experiment 1, N=1/78 in choice experiment 2, N=1/40 in choice experiment 3). In

addition, one trial of the no-choice experiment 3 was excluded because the male lacked genitalia. For each experiment, we counted the number of trials in which each of the distinct mating behaviours occurred (for males: palpation, mounting, attempting, copulation; for females: rejection upon male attempt or not, Fig. 1). Since palpated females only occasionally remained unmounted, we did not analyse this behaviour any further. For each trial, we registered how far the male progressed in the chain of sequential mating behaviours (palpation, attempt, or copulation). In choice experiments this was recorded for each of the two females offered. For each female we calculated the amount of time in seconds between each pair of sequential events: time until first palpation (O-P), from first palpation to first attempt (P-A), and from first attempt until first copulation (A-C). In experiments where different male groups were used (choice experiment 1 and 2), we first compared their behaviour and female rejection behaviour towards them. If no significant differences were found, data of all males were pooled for further analysis on female groups.

Generally, Chi-square tests were used to compare frequencies of trials. To examine which factors affected the probability of a specific behaviour to occur in a trial, we fitted generalized linear models (GLMs) with a Binomial distribution and a logit link function. We included a relevant selection of the following factors as explanatory variables: male size, female group, female size, female rejection behaviour, and degree of winglessness. We first fitted fully saturated models and then stepwise removed explanatory variables until the minimal adequate models were found. Variable significance levels were determined from Chi-square tests on changes in the deviance following removal of a variable. In the same way, we applied GLMs with a Gamma distribution and an inverse link function to test whether these factors and (only in the no-choice experiment) the incidence of mating influenced the time lapse between sequential behaviours. However, in this case significance levels are reported from F-tests on the change in deviance to deal with overdispersion (Crawley 2007). For the no-choice tests, we pooled female groups with less than five cases to perform this analysis. In the choice tests, the timing of events was only analysed for the mated female. In choice experiment 1 we only used replicates where both brothers mated.

The relation between body parts and the frequency of these being palpated was only analysed for the no-choice experiment. We compared the frequency of palpation between body parts and female group by Generalized Mixed Effect Models (GLMMs) with a Poisson distribution and a log link function with body part nested within trial as random factor. Significant levels are reported from Chi-square tests on changes in the deviance following removal of a factor (level). Finally, we tested the effect of sex, group, and family (when applicable) on body size by a Student's t-test or an Analysis of Variance (ANOVA).

Results

No-choice experiment

Figure 3 shows how far males progressed in the chain of behaviours towards mating in the no-choice experiments, expressed by the frequency of trials. Although female groups significantly differed in body size (ANOVA, $F_{6,180}=7.308$, $p<0.001$, with fully winged wingless and wildtype females being smaller than the wingless phenotypes), female body size did not affect the probability of male attempt (GLM, $\chi_1^2=1.381$, $p=0.240$). Female group, however, significantly affected the probability of an attempt (GLM, $\chi_1^2=73.949$, $p<0.001$): the chance of a female being attempted significantly increased with the following groups: $WL_0 < WL_2+WL_3 < WL_4 < WL_5+WL_F+WT$. Similarly, copulation did not depend on female body size (GLM, $\chi_1^2=0.057$, $p=0.811$), but significantly increased with elytron length in the groups $WL_0 < WL_2+WL_3 < WL_4+WL_5 < WL_F+WT$ (Fig. 3). Female rejection occurred in all female groups except WL_0 , but never prevented mating (Fig. 3).

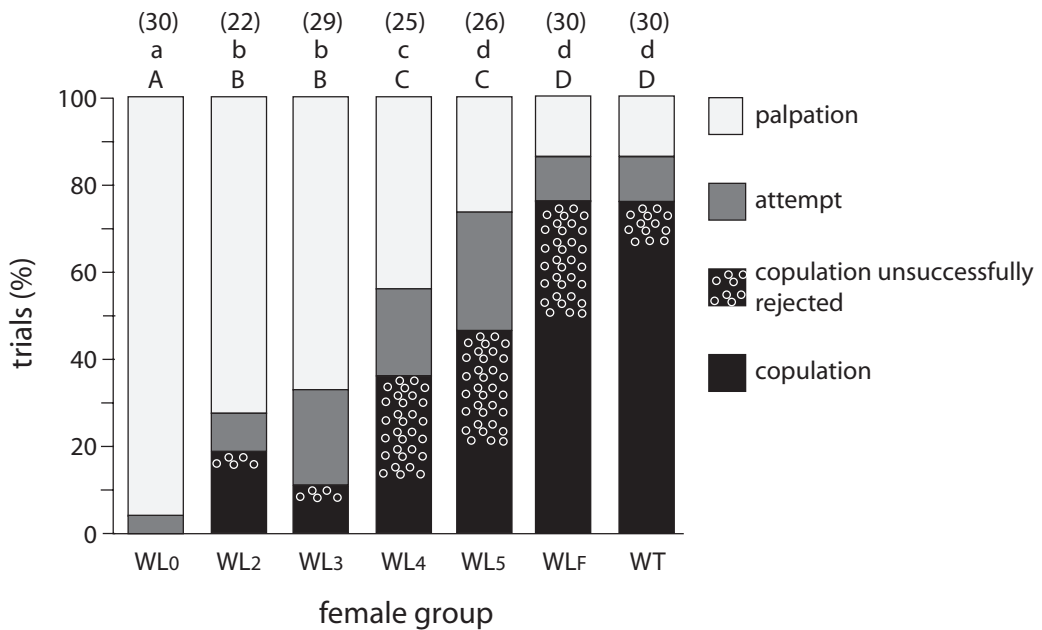


Figure 3. Frequency of trials in which males displayed mating behaviour when offered a single female. Columns represent female groups; six wingless genotypes were used, increasing in elytron length from WL_0 to WL_F (see material and methods), and one group of females wild-type for the wingless trait (WT). Stacks represent behaviour types that males display in fixed sequence from light to dark. Circles indicate female rejection upon male mating attempt. Numbers between brackets represent the number of replicates. Female groups with different letters significantly differ in the number of trials in which a behaviour was displayed (GLM, $p<0.05$), where lower case letters and capitals stand for the trials with attempts and copulations, respectively.

Further analysis of the frequency of actions within trials revealed that the palpation frequency was higher at the front of the female body than at the rear (GLMM, $\chi_1^2=25.672$, $p<0.000$). Palpation frequency within trials significantly differed between female groups (GLMM, $\chi_1^2=42.668$, $p<0.001$), with females bearing longer elytra receiving fewer palpations: $WL_F+WT < WL_4+WL_5 < WL_0+WL_2+WL_3$. Females phenotypically wingless were more often attempted after palpation on the front of the body than after palpation at the rear ($WL_0+WL_2+WL_3$: $\chi_1^2=6.813$, $p<0.010$; WL_4+WL_5 : $\chi_1^2=5.792$, $p<0.050$). For phenotypically winged females, attempts were equally often following palpation at the front, and following palpation at the rear of the body (WL_F+WT : $\chi_1^2=0.734$, $p=0.392$).

Analysis of the time intervals between consecutive behaviours showed that none of these was affected by female size (GLM; 0-P: $F_{1,179}=0.769$, $p=0.382$; P-A: $F_{1,91}=0.268$, $p=0.606$; A-C: $F_{1,64}=0.000$, $p=0.999$). There was a trend for intervals to shorten with increased in elytron length (Fig. 4A), but differences between groups were never significant (GLM; 0-P: $F_{1,185}=1.323$, $p=0.249$; P-A: $F_{1,90}=1.0826$, $p=0.376$; A-C: $F_{1,68}=1.360$, $p=0.258$). The only factor significantly affecting the time until palpation and time between palpation and attempt was copulation, with shorter intervals for copulated females than those not copulated (GLM; 0-P: $F_{1,186}=6.335$, $p<0.050$; P-A: $F_{1,92}=6.495$, $p<0.050$) (Fig. 4A). Female rejection behaviour upon male attempt significantly delayed the timing of copulation ($F_{1,69}=11.816$, $p<0.010$) (Fig. 4B).

Summarising, the incidence of mating increased with female elytron length, whereas the palpation frequency per trial decreased.

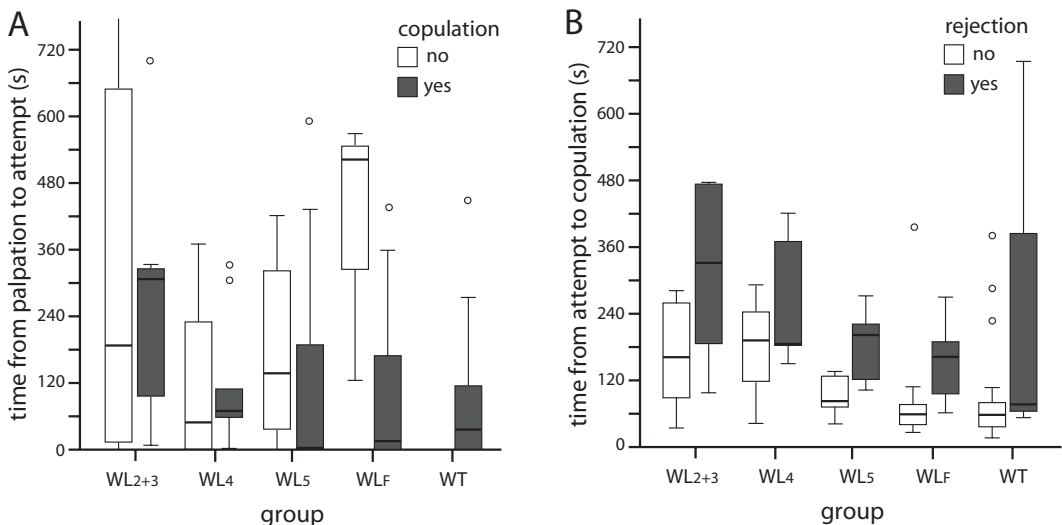


Figure 4. Timing of events preceding copulation per female group. Boxes represent interquartile ranges (IQR), covering 50% of all data points, thick lines in boxes indicate median values, and whiskers show the range of values that fall within 1.5 IQR of the box. Dots represent outliers. A) Time from palpation to attempt is significantly shorter for copulated females (grey boxes) than for females not copulated (white boxes). B) Time between attempt and copulation is significantly prolonged in females that reject (grey boxes) compared to those that do not reject (white boxes).

Choice between wingless and winged sisters

To assess putative pleiotropic effects of the wingless allele on male mating behaviour, we first compared the behaviour of winged (HET) to that of wingless (WL) males. Figure 5 shows the number of trials in which males palpated, attempted and copulated both females, only the winged (HET) female, or none of the females (males never displayed these behaviours exclusively with the wingless (WL) female). These numbers did not significantly differ between the two male phenotypes (all: $N=32$; palpation: $\chi_1^2=1.237$, $p=0.266$; attempt: $\chi_1^2=3.017$, $p=0.221$; copulation: $\chi_1^2=2.413$, $p=0.120$). Both male phenotypes only copulated with winged females. The number of females that showed rejection behaviour upon male attempt also did not significantly differ between the male phenotypes ($N=5$ out of 25 attempts by winged males, $N=7$ out of 29 attempts by wingless males, $\chi_1^2=0.000$, $p=1.000$), and only rarely prevented mating ($N=2$ for winged males; $N=1$ for wingless males).

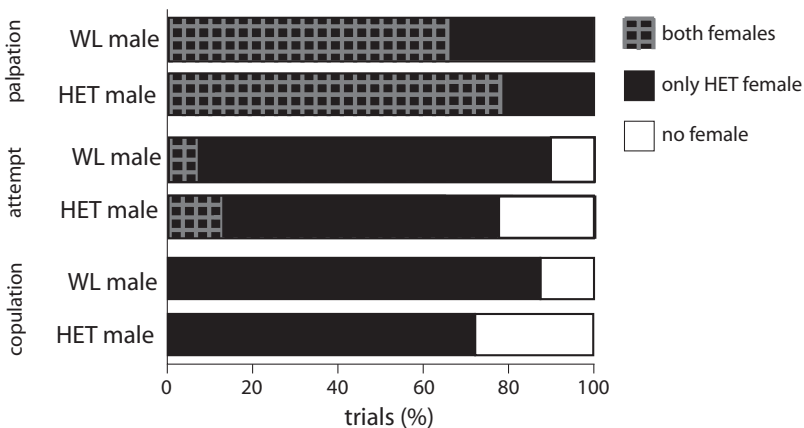


Figure 5. Frequency of trials where either a winged (HET) or wingless (WL) male interacted with winged (HET) and wingless (WL) females when offered both in a choice test ($N=32$). Winged and wingless males behave similarly, but wingless females are less often palpated, attempted and copulated than their winged sisters.

Winged and wingless males showed similar frequencies of mating behaviour, and the timing of events preceding mating with the winged female was the same. There was no significant difference in the time until they first palpated the winged female (GLM, $F_{1,41}=3.711$, $p=0.061$), and the time it then took them until their first attempt (GLM, $F_{1,41}=0.419$, $p=0.521$) (Fig. 6). The time from attempt to copulation was significantly affected by the interaction between male phenotype and female rejection (GLM, $F_{1,39}=5.853$, $p=0.020$), such that female rejection delayed copulation by wingless males, but not by winged ones. However, this did not result in significant differences between the male phenotypes in mean time from attempt to copulation (GLM, $F_{1,41}=0.0287$, $p=0.866$) (Fig. 6).

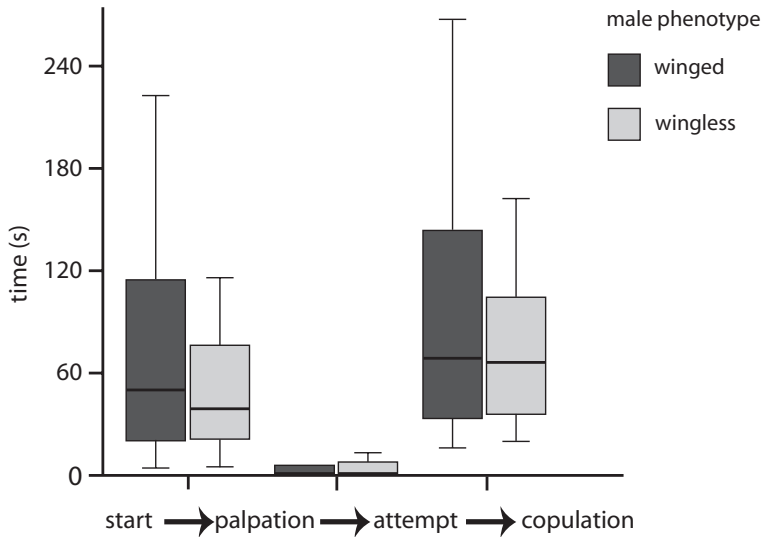


Figure 6. Timing of behaviours by winged (HET, dark bars) and wingless (WL, light bars) males preceding copulation of the winged (HET) female in a choice test with a wingless female (WL, which was never mated) (N=21). Boxplots as in Fig. 3.

The total time from first palpation until copulation was neither affected by male phenotype (GLM, $F_{1,40}=0.368$, $p=0.547$), nor female rejection (GLM, $F_{1,41}=0.292$, $p=0.262$), or their interaction (GLM, $F_{1,39}=3.526$, $p=0.068$).

Since winged and wingless males showed equal patterns in the frequency and timing of behaviours, the male groups were pooled, and we proceeded with the analysis of female effects. The interaction of wing phenotype and sex had a significant effect on body size (ANOVA, $F_{1,195}=6.847$, $p<0.01$): wingless beetles were wider than their winged brothers or sisters, but the difference was larger for males than for females. Therefore, male and female body size were both included in the analyses. The average degree of winglessness (DWL) class of wingless females was 2.66 ± 1.10 (mean \pm SD). In most trials both the winged and the wingless female were palpated (Fig. 5), and they were equally likely to be palpated first ($\chi_1^2=0.563$; $p=0.453$). However, winged females were palpated in more trials than their wingless sisters ($\chi_1^2=19.920$, $p<0.000$): all winged females, but not all wingless females were palpated. The probability that a wingless female was palpated was affected by her size, with smaller females having a higher chance (GLM, $\chi_1^2=5.934$, $p<0.050$), but not by her DWL (GLM, $\chi_1^2=0.696$, $p=0.404$), or male size (GLM, $\chi_1^2=1.211$, $p=0.271$). Consecutively, winged females were more often attempted ($\chi_1^2=73.026$, $p<0.000$) and copulated ($\chi_1^2=85.247$, $p<0.000$) than their wingless sisters: the majority of winged females were attempted and mated, whereas only a few wingless females were attempted (N=6), and although they were never observed to reject in response, none were copulated (Fig. 5). The probability of a female being attempted did

not depend on their size or the male's size, and neither of these factors nor rejection significantly influenced chances of copulation.

Choice between winged females with one wingless allele or without

The control experiment using winged beetles with the wild-type (WT) and the heterozygote (HET) genotype on the wingless locus revealed no differences in behaviour between the genotypes in either sex with respect to the frequency of interactions and the timing of behaviour, except that WT males spent generally less time in palpation of the female that was eventually mated (GLM, $F_{1,56}=5.323$, $p<0.050$).

Choice between winged females with two wingless alleles or without

Males were offered two winged females, one with the wingless genotype (WL_r), and one with the wild-type genotype at the wingless locus (WT). Females did not differ in size (t-test, $t=0.210$, $p=0.835$). When both females genotypes were palpated in a trial, both were equally likely to be palpated first by the male ($\chi_1^2=2.286$; $p=0.131$). There was no significant difference in the total number of trials where palpation occurred ($\chi_1^2=0.970$, $p=0.325$), but attempts to mate were fewer with wingless females than with wild types ($\chi_1^2=7.811$, $p<0.01$), and they were less often copulated ($\chi_1^2=10.125$, $p<0.01$) (Fig. 7). Both female genotypes responded equally often with rejection behaviour upon male attempt (WT: 10/27; WLF: 1/10; $\chi_1^2=1.423$, $p=0.233$), but rejection never prevented mating. The timing of behaviour preceding mating did not differ significantly between the female genotypes.

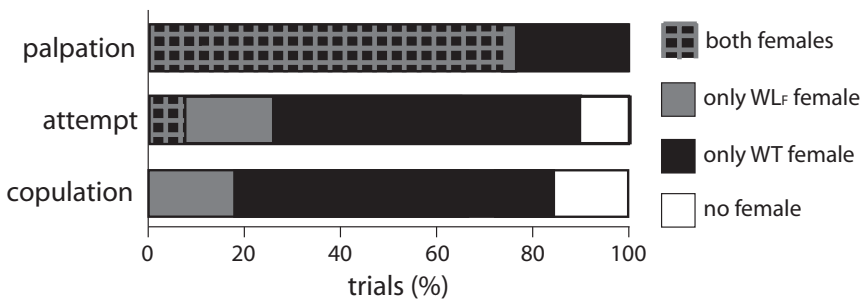


Figure 7. Percentage of trials in which wild-type males palpated, attempted, and copulated winged females with the wild-type genotype (WT) and the wingless genotype (WL_r), when offered simultaneously in a choice test (N=38).

Discussion

Winglessness affects mating in *A. bipunctata*

We observed strong effects of winglessness on mating in *A. bipunctata*. Specifically, winglessness in females affected male mating behaviour. Thus, males offered a single conspecific female never copulated with her when she had no elytron tissue (WL_0), but attempts and copulations increased with female elytron length, and fully winged but genetically wingless females (WL_r) were as often copulated as winged wild types (WT) (Fig. 3). When given the choice between a winged and a wingless sister, successful males always copulated with the winged one (Fig. 5). When offered two winged females, one genetically wingless and the other wild-type, the latter were more frequently copulated (Fig. 7).

We found no evidence for pleiotropic effects of the wingless allele on cues for mating that could explain these results. First, our behavioural essays revealed no effects of the wingless allele on mating behaviour in either sex: males and females possessing one or two copies of the wingless allele behaved similarly to wild types. This also justifies the use of winged beetles heterozygous for the wingless trait instead of wild types in choice experiments 1 and 2. Second, we found a pleiotropic effect of the wingless allele on body size, a trait that has been associated with mating preference (Tomlinson et al. 1995; Yasuda and Dixon 2002). Wingless beetles were on average larger than winged conspecifics. However, female size was never found to affect the chance a female was mated significantly. Third, a preliminary analysis of the composition of cuticular hydrocarbons from the pronotum, elytra, and abdomen of winged and wingless females revealed that only 4% of the total variation was explained by the different genotypes, whereas 40% was explained by variation between body parts. These results indicate that winged and wingless morphs differ hardly in CHC composition. This is in line with the hypothesis that these CHCs act as species-specific recognition cues (Hemptinne et al. 1998), and not as recognition cues of genotypes within species.

In sum, the wingless allele does not seem to affect behavioural, visual or olfactory mating cues. It is, therefore, likely that the observed effects of winglessness in females on male mating behaviour can be entirely attributed to the deviations in the female elytra. Below we initially discuss which mating cues provided by the elytra can explain the reduced mating frequency with wingless females. We then examine implications for wingless beetles in the wild and for their use as natural enemies in biological control programs.

Elytra and chemical mating cues

Although elytra bear CHCs that act as species-specific recognition cues eliciting male mating behaviour (Hemptinne et al. 1998), we reason that the lack of elytra tissue does not impair species recognition since it is unlikely that the chemical mating cues are associated exclusively with the elytra. First, the insect literature reports that CHCs are part of the cuticular waxes that cover

entire insect bodies (Howard and Blomquist 1982). Second, we analysed the frequency with which different body parts were palpated, and how often this behaviour was followed by copulation. Whereas Hemptinne et al. (1998) noticed that elytra were often contacted first by males, we found that initially in courtship, the anterior portion of females was generally palpated more frequently than the posterior. Palpation on the front of the female body was as frequently followed by copulation attempts as palpation on the elytra of winged and fully-winged wingless females. This implies that the front of the body also includes the species recognition cue(s) that elicits copulation attempts. Our observation that palpation frequency within a trial increased with shorter female elytron length is probably a direct consequence of the decreasing chance of being mated, and not a sign of lack of recognition. The less often, or later an attempt is made to copulate, the more time is spent on palpation. Third, in a preliminary analysis of the CHC composition in *A. bipunctata*, CHC composition of the elytra did not significantly differ from that of the abdomen (unpublished data). When males palpate the dorsal surface of wingless females, they may contact the abdominal surface instead of the elytra but these body parts probably provide the same chemical cues. Research on other species of beetle indicates that males will mate with any material, as long as they bear the active compound (Fukaya et al. 1996; Yasui et al. 2003; Sugeno et al. 2006). In summary, we assume that body parts other than the elytra can provide the olfactory species recognition cue, and therefore elicit mating behaviour in *A. bipunctata*. We then assume that wingless females will be recognized as potential mating partners upon palpation. To confirm this, the active compound(s) in the CHC blend will need to be identified for *A. bipunctata* (Howard and Blomquist 2005), and its presence and abundance compared between winged and wingless specimens.

Elytra and visual mating cues

For *A. bipunctata*, data on visual mating cues potentially provided by elytra are scarce. Hemptinne et al. (1996) showed that contact with female elytra of *A. bipunctata* induced intensified searching behaviour by *A. bipunctata* males. In contrast, such elytra washed in chloroform, did not elicit this response. Female elytra of the ladybird *Coccinella undecimpunctata* (L.), similar in size and colour to those of *A. bipunctata*, also failed to elicit this response. This implies that contact sex pheromones on elytra may be more important than visual cues. Studies of certain populations and laboratory stocks have suggested that female preferences can occur for male melanic colour morphs in *A. bipunctata* (Majerus et al. 1982). However, such preference may not be mediated by the colour pattern itself since the different colour morphs differ in many other traits (De Jong et al. 1996). Moreover, this form of preference has not been found for the Utrecht localities (Brakefield 1984c). In our choice experiment with winged and wingless sisters, only winged females were copulated although they were not any more likely to be the first to be palpated by the male. This strongly suggests that the shape of the elytra is not a visual cue for species recognition or mating in this species.

Elytra and tactile mating cues

Our behavioural assays strongly indicate that the female elytra provide a tactile cue for male mating behaviour in *A. bipunctata*. We put forward three potential mechanisms that are not mutually exclusive. With a few exceptions, all wingless females palpated were mounted, so elytra do not seem a prerequisite for mounting. However, once mounted, males retracted their genitalia again frequently when attempting to copulate with a wingless female, but this was seldom observed with winged females. The chance that a male attempts to copulate with a wingless female increased with increasing elytron length (Fig. 3). In the no-choice experiment, wingless females in DWL class 5 with more than $\frac{3}{4}$ of the abdomen length covered by the elytra were attempted as frequently as fully winged wingless females and wild types, but were copulated less frequently than either of the latter in the no-choice experiment. There was also a non-significant trend for decreased time from palpation to attempt, and from attempt to copulation, with increasing elytron length.

There is no evidence that male *A. bipunctata* express mate preference. Thus, it is more likely that these observations are related to the ability for males to mate with particular phenotypes, and the properties of the elytra that could facilitate this in a physical way. Our first hypothesis is that the outline of the elytra guides a mounted male to the precise location of the female genitalia. The female genitalia are located at the tip of the abdomen, and in wild types, close to the caudal end of the elytra (Fig. 8). This is further supported by observations of some males in this study trying to insert their genitalia underneath the pronotum shield and the end of the truncated elytra of wingless females halfway along the abdomen. In some other species of beetles, mating males have been shown to use tactile cues for orientation. Males of a longicorn beetle positioned themselves on female models based on the shape and size of the model (Fukaya et al. 1996), and males of a staphilinid beetle (Peschke 1979), and a anobiid beetle (Ward 1981) were found to use female setae for orientation. Second, elytra could provide the male with grip while mating. This was also as observed in *Pidonia* longicorn beetle species (Tanigaki et al. 2007). Indeed, mating *A. bipunctata* males were sometimes observed to hold the edges of the female elytra (Fig. 8), for example when they could not stabilize themselves because there was no platform for their most caudal pair of legs to grip.



Figure 8. A wingless male copulates with a winged female while holding the edge of her elytra.

Mating by wingless *A. bipunctata* in the wild

We here consider the implications of our findings for the consequences of the wingless allele for mating in the wild. Wingless phenotypes rarely occur, but the wingless allele could be maintained for some time in winged individuals heterozygous for the wingless locus. Our results demonstrate that mating is not affected in such heterozygotes. Therefore, the wingless allele will only affect mating in homozygotes with a wingless phenotype. In our experiments with phenotypically wingless individuals, winglessness in females, but not in males, obstructed mating. However, all experiments were carried out in a restricted area preventing flight. Under natural conditions where flying is not hindered, the ability to fly will strongly affect the probability of mating of both sexes. Flight might be required to migrate to sites suitable for reproduction, since beetles switch between several different host plants throughout the season (Brakefield 1984a). Thus, wingless females might have difficulties finding or reaching areas with plants hosting aphid prey, and wingless males might have the same problem in localising and reaching these areas with potential mating partners. We, therefore, expect wingless beetles of both sexes to have a lower chance of mating with in the wild than their winged conspecifics. If males contact wingless females in the wild after all, it is difficult to predict the chances of copulation. On the one hand, winged females are likely to be favoured when both phenotypes are present at the same site. Therefore, we expect that female density is an important factor in determining the chance that wingless females are mated. On the other hand, we expect females to be more effective in rejecting males in open environments in nature than in our experiments where rejection rarely prevented males from copulation. For example, flipping over might then result in dropping off the host plant and release of the male, and winged females will consequently be able to flee from mating by flying away. In this respect, once localized by males, wingless females might be easier to mate with.

The evolution of winglessness in *A. bipunctata*

How does mating affect the evolution of winglessness in *A. bipunctata*? Mating frequency is a key factor in sexual selection, so if wingless individuals in the wild indeed mate less frequently than winged ones as suggested above, this may affect the evolution of the wingless allele. *Adalia bipunctata* is highly promiscuous (Haddrill et al. 2008). Polyandry is shown to increase offspring number in *A. bipunctata* (Haddrill et al. 2007), and is generally considered advantageous by increasing offspring quality (e.g. Zeh and Zeh 1997; Jennions and Petrie 2000). Wingless females, if indeed mating less frequently, may have a disadvantage in this respect.

However, female resistance behaviour suggests that mating is costly in *A. bipunctata* (Perry et al. 2009). Though no direct cost of mating in terms of reduced longevity was found for females mating multiply (Perry and Rowe 2008), other direct or indirect costs could play a role. For example, indirect costs may include the risk of sexually transmitted diseases which can have a large impact

on population dynamics (Knell and Webberley 2004). The highly virulent parasitic mite *Coccipolipus hippodamiae* (McDaniel & Morrill) is common in *A. bipunctata*, lives underneath their elytra, and is predominantly transmitted during mating (Hurst et al. 1995; Webberley et al. 2004). It does not affect mating behaviour of its host (Webberley et al. 2002), but has severe fitness effects by causing female sterility and deleteriously affecting males, too (Hurst et al. 1995). The ladybird's promiscuity thus promotes sexual transmission of this parasite (Webberley et al. 2004; 2006). Wingless females may have a lower chance of becoming infected when mating less frequently, and by having less elytron tissue to host the mites. They could have a selective advantage in this context. Such benefits could partly outweigh the major disadvantages of winglessness (Ueno et al. 2004), and thus promote some local spread of the wingless allele in the population. To test this hypothesis, wild populations of *A. bipunctata* should be screened for the frequency of the wingless allele and the incidence of infection with the parasite, and laboratory experiments used to examine the relationship between elytron length and the transmission rate of parasites.

Implications for the use of *A. bipunctata* in biological control

Adalia bipunctata is sold as a biological control agent of aphid pests in greenhouses and urban trees. It has been shown that wingless morphs of this predatory ladybird can be more effective in aphid control because their residence time on the host plant is longer (Lommen et al. 2008). Ease of mass-rearing of wingless morphs is a prerequisite for commercial companies to consider them as a new product. Our experiments should not necessarily raise concerns for mass-rearing since the limited time frame in which the behaviour assays were performed has probably strongly affected the results. When wingless ladybirds are held in groups over a longer period, matings are observed frequently, and they reproduce well. This indicates female elytra facilitate, but are not essential for, mating.

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