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Seasonal cycles of assortative mating and reproductive behaviour in polymorphic populations of *Harmonia axyridis* in China

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Abstract. 1. We observed native populations of *Harmonia axyridis* (Pallas) around Beijing, China, over 2 years and performed choice and no-choice mating tests between melanic and succinic (non-melanic) beetles in the laboratory.

2. Succinic phenotypes outnumbered melanics by 5:1 in autumn, but melanics became equally abundant in spring, supporting previous inferences that melanism is advantageous in winter, but costly in summer.

3. Female *H. axyridis* expressed mate preference overtly, by rejecting less-preferred phenotypes, and cryptically, by retaining their eggs for longer periods after matings with less-preferred males, ostensibly to replace their sperm.

4. Succinic pairs formed more quickly in the spring generation, and melanic pairs in the autumn, and the time to copula was affected by both male and female phenotype. The strength of mate preference was contingent on female phenotype, suggesting melanic alleles had pleiotropic effects.

5. Whereas pair formation was under female control, the duration of copula was under male control and lasted longer in the autumn generation than in the spring. Copulations in the choice test tended to be shorter between similar phenotypes, suggesting that males invested more in dissimilar females when alternative mates were available.

6. Although spring and autumn generations were raised under identical conditions, significant contrasts were observed in their reproductive behaviour.

7. Two alternative hypotheses are advanced to explain why gender-specific reproductive behaviours might vary between generations: maternally-mediated epigenetic factors that influence the expression of genes in progeny as a function of maternal environment, and linkage disequilibria among alleles that cycle in frequency seasonally as a function of assortative mating.

Key words. Coccinellidae, female choice, mate preference, melanism, polymorphism, seasonality, sperm competition.

Introduction

Melanism is a taxonomically ubiquitous phenomenon that refers to the occurrence of phenotypes with especially dark coloration relative to typical conspecifics. It has long been a topic of interest among naturalists, because of its apparent effects on life history, reproduction, crypsis, and overall survival. The ecological significance of melanism has been studied in fish (Horth, 2003), reptiles (Clusella-Trullas *et al.*, 2008; Lorioux *et al.*, 2008) birds (Roulin, 2007), and mammals (Majerus & Mundy, 2003), but perhaps most notably in insects. Indeed, the study of industrial melanism in the peppered moth, *Biston betularia*, remains a classic example of evolution being witnessed in action (Kettlewell, 1955, 1956; Majerus, 1998).

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Among the Coccinellidae, colour polymorphisms that include melanistic forms have a long history of study (Dobzhansky, 1933; Tan, 1946; Komai, 1956; Creed, 1975; Muggleton *et al.*, 1975a). Multiple alleles are involved in the inheritance of colour patterns in these beetles (Hodek & Honěk, 1996), giving rise to a range of both melanic and succinic (non-melanic) phenotypes. The relative frequencies of melanic and succinic forms may vary both geographically (Timofeeff-Ressovsky, 1940; Brakefield, 1984a) and seasonally (Tan, 1949; Rhamhalinghan, 1988; Osawa & Nishida, 1992) and are often maintained by sexual selection (e.g. Muggleton *et al.*, 1975b; O'Donald & Majerus, 1984). However, no single ecological hypothesis for the selection and maintenance of colour polymorphisms appears to have universal applicability among geographically isolated populations or across taxa (Hodek & Honěk, 1996).

Harmonia axyridis (Pallas) (Coleoptera: Coccinellidae) is a highly polyphagous ladybird beetle and a strongly invasive species in many regions, where it has been introduced either purposely or accidentally (Koch, 2003; Soares *et al.*, 2008). Within its native range in China, *H. axyridis* presents a wide range of elytral colour patterns (Pang *et al.*, 2004; Yu, 2008) and also exhibits regular seasonal shifts in the relative abundance of these phenotypes (Yuan *et al.*, 1994; Jing & Zhang, 2001; Jiang *et al.*, 2008).

The numerous elytral patterns in *H. axyridis* can be divided into two general phenotypes on the basis of background coloration: melanic (dark) and succinic (light) which result from alternative alleles at a single locus (Tan & Li, 1934; Tan, 1946; Anonymus, 1975; Ueno *et al.*, 1998). Elytral colour is a function of the distribution of two types of pigments, the dark pigment melanin and various carotenoid derivatives that range from cream through to shades of yellow, orange, and red (Britton *et al.*, 1977a). Although much of the intensity of orange and red is a function of diet, some of the pale carotenoid compounds are apparently produced *de novo* within the beetles (Britton *et al.*, 1977b). Since melanin masks lighter pigments and its deposition is an active process, melanic alleles tend to be dominant.

Elytral colour is not a trivial trait and may have consequences for an individual's biological performance and life history. For example, the thermal consequences of melanism have been directly measured (Brakefield & Wilmer, 1985; Rhamhalinghan, 1990). Stewart and Dixon (1989) argued that large ladybird species should not evolve melanic forms based on the established relationships between body size, rate of excess heat loss, and the thermal properties of dark elytra. Seeking to test the theory of thermal melanism, Brakefield (1984b) observed that melanic *A. bipunctata* emerged earlier from pupae in the spring and dispersed, reproduced, and died earlier, than non-melanic forms. Soares *et al.* (2001, 2005) found that two contrasting phenotypes of *H. axyridis* differed not only in voracity and body weight, but also in fecundity, longevity, and prey suitability. Bezzerides *et al.* (2007) showed that alkaloids concentrations in *H. axyridis* were negatively correlated with the size and intensity of melanic spots, particularly in females, and concluded that non-melanics were better chemically defended. True (2003) discussed insect melanism in the context of other life history traits, and suggested molecular mechanisms operating at the level of either gene regulation or protein coding could lead to pleiotropic effects on other traits with important biological functions. Srivastava and Omkar (2005) studied a melanic morph of *Coccinella septempunctata* L. and found that melanism was associated with increased longevity, fecundity, and mate preference in this species, despite the relative rarity of melanics in the field. Similarly, Berkvens *et al.* (2007) measured differences in performance between melanic and succinic phenotypes of *H. axyridis*, and concluded that the latter had greater nutritional plasticity. Thus, melanism in coccinellids may correlate with a range of physiological attributes that can be advantageous under particular circumstances, but not others.

Assortative mating refers to the tendency of females of one phenotype to mate preferentially with males of the same or different phenotype. Assortative mating based on elytral colour has long been recognised in *A. bipunctata* (Brakefield, 1984c). Female preference for male colour is heritable in this species and responds to artificial selection (Majerus *et al.*, 1982; O'Donald & Majerus, 1992). O'Donald and Majerus (1984) demonstrated that assortative mating in *A. bipunctata* produced negative frequency-dependent selection capable of stabilizing a colour ploymorphism, and O'Donald and Muggleton (1979) used modelling to show that this process could stabilise polymorphisms at a wide range of equilibrium frequencies. Given the seasonal shifts in female preference observed by Osawa and Nishida (1992) in Japanese populations of *H. axyridis*, we hypothesised that environmentally dependent assortative mating could be responsible for the seasonal cycles of abundance of melanic *H. axyridis* observed in China (Jiang *et al.*, 2008). We hypothesised that mate choice could be expressed both overtly, via frequency of male rejection events and duration of copula, and cryptically, via differential egg retention time after copulation.

In Beijing, aphid populations peak during cool weather in spring and autumn, but become scarce during the hot summer months (May–August) when maximum daily temperatures average above 35 °C (Chen *et al.*, 1987). This results in two relatively discrete generations of *H. axyridis*: one emerging as adults in spring that aestivates in summer and breeds in the autumn (hereafter referred to as autumn adults), and one emerging as adults in autumn that overwinters in hibernation to breed in the spring (hereafter referred to as spring adults). We use the term 'phenotype' as shorthand for colour morph, except where otherwise specified. The objectives of the present study were to: (1) characterise seasonal cycles of polymorphism in native *H. axyridis* populations around Beijing, China; (2) determine if assortative mating occurred and if mate preference criteria shifted seasonally; (3) characterise the mechanism(s) of assortative mating and; (4) test for broader effects of male and female phenotype on reproductive behaviour. For these purposes, seasonal field observations were made in four localities over 2 years, and choice and no-choice mating tests were performed under controlled laboratory conditions with both spring and autumn generations.

Methods

Seasonal variation in relative abundance of phenotypes

Observations on the relative abundance of melanic and succinic phenotypes of *H. axyridis* were made in four rural

locations: (1) An apple orchard in Pinggu County (northeastern Beijing, 40° 05′N, 117° 04′E); (2) a private vegetable farm in Yanqing County (northwestern Beijing, 40° 27′N, 115° 57′E); (3) a wild peach woodland in Lingshan, Mentougou County (western Beijing, 40° 11′N, 114° 42′E), and (4) Shidu, a natural mountain conservation area in Fangshan County (southwestern Beijing, 39° 37′N, 115° 33′E). Summer observations were made from July to August, and autumn observations from October to December in both 2006 and 2007. Observations were taken on sunny, windless days between the hours of 09.00 and 15.00 once every 2 weeks for a total of five independent observations at each site in each seasonal period. The numbers of melanic and succinic *H. axyridis* adults were tallied visually by counting them on plants. In addition, collections were made with a motorised vacuum sampler on the vegetable farm, in order to obtain beetles obscured in dense herbaceous vegetation. All phenotypes in which the background colour of the elytra was dark were categorised as melanic; all phenotypes in which the background colour was some shade of yellow, orange or red were categorised as succinic. The equilibrium frequency of the melanic allele in the population was calculated as $1-\sqrt{f_s}$, where f_s is the mean frequency of the succinic phenotype.

Collection and maintenance of the stock colony

Adult *H. axyridis* used to establish laboratory colonies were collected from a citrus orchard in Beijing, China (40° 14′ 33″N, 116° 13′ 39″E) in August 2006 and again in April 2007. These collections were timed to coincide with the onset of autumn and spring when natural populations were most abundant. After each collection, a colony was established in the laboratory under controlled environmental conditions (26 ± 1.0 °C, LD 16:8 h, and 70% RH). Groups of 45–55 adult beetles were initially confined in aluminum frame cages (30.0 × 15.0 × 20.0 cm), covered with sheer nylon fabric and fed an *ad libitum* diet of *Aphis craccivora* Koch, provided fresh daily on excised bean shoots. The aphids were reared in continuous culture on broad bean, *Vicia faba* L., in a greenhouse under natural lighting.

For purposes of reproduction, pairs of beetles were transferred to small plastic cages (25.0 × 15.0 × 10.0 cm), five pairs per cage, and provisioned daily with fresh *A. craccivora* on bean shoots. Strips of white filter paper (10.0 × 3.0 cm) were placed in the cages to serve as an oviposition substrate. Eggs were collected at 8-h intervals and held in plastic Petri dishes (9.5 cm diameter) until hatching. Newly hatched larvae were transferred to clean Petri dishes, three per dish, using a fine Chinese writing brush. Larvae were fed *A. craccivora* daily until they reached the third instar, whereupon they were transferred to larger cages (65.0 × 48.0 × 48.0 cm), 70 per cage, and fed until pupation.

Newly emerged adults were sexed and divided into four groups according to sex and colour: melanic males, melanic females, succinic males, and succinic females. Adults of each grouping were held in plastic cages (as above), 10 per cage, and fed fresh *A. craccivora* daily for at least 15 days prior to use in copulation experiments.

Mating experiments

Mating experiments were conducted in late summer (August to September) in 2006 and repeated in late spring (April to May) in 2007, with adult beetles that were reared in the laboratory from individuals field-collected in the previous season. Phenotypes were selected for experiments as follows (see Tan, 1946 for plate of elytral patterns). Succinic individuals used (forma *succinea*) had anywhere from eight to 12 black spots on a yellow or orange background; melanic individuals had either two (*conspicua*) or four (*spectabilis*) red spots on a black background. No immaculate or intermediate forms (e.g. *aulica*, *intermedia*, *tripunctata*) were used.

The experiments consisted of a choice mating test and a no-choice mating test, both conducted on a laboratory bench at 22 ± 1 °C, 70% RH, under fluorescent lighting. In the choice test, 15-day-old virgin adults of each colour/sex combination (n = 60 beetles) were placed in a 1-litre, wide mouth glass Mason jar with abundant prey provided on excised bean shoots. The mouth of the jar was covered with a fine screen to contain the beetles until mating pairs formed. Copulating pairs were removed sequentially as they formed. The time of each mating was recorded and each pair was immediately transferred to a plastic Petri dish (5.5 cm diameter) and provided with aphids on a bean shoot. The colour morph of each beetle was recorded and a pair of beetles of similar colour and sex was immediately added to the jar to maintain constant encounter rates among phenotypes throughout the experiment. The experiment continued until ten pairs of each sex/colour combination were obtained ($♀_m + ♂_m$, $♀_m + ♂_s$, $♀_s + ♂_m$ and $♀_s + ♂_s$). When a copulating pair separated, the copulation time was recorded and the male removed at once. Females were then isolated in Petri dishes (as above) and fed an *ad libitum* diet of *A. craccivora* until they laid eggs. The following data were recorded for each female: the time to copula (the period from placement of beetles in the jar until onset of copulation), the duration of copula (the period from onset of copulation until pair separation), and the egg retention period (the period from termination of copula until first oviposition).

In the no-choice mating experiment, we isolated arbitrarily selected pairs of 15-day-old virgin adults in plastic Petri dishes (as above) with ten replicates of each of the four phenotype combinations. After copulation, males were removed and females were fed aphids *ad libitum* in isolation, until eggs were laid. All data were recorded the same as in the choice test.

During both choice and no-choice experiments, we used a digital video camera (Sony NP–F750 Konan, Minato-ku, Tokyo, Japan) to record mating behaviour. We mounted two video cameras to record sexual interactions, one directly above the container and the other facing the side. We began recording whenever a male began to chase a female, and continued until copulation. Mating was considered successful when abdomenal shaking by the male was observed, a reliable indication of insemination (Obata, 1987). The video records were used to characterise female rejection behaviour and tally the frequency of rejection events. The latter were defined as recognizable evasive or resistant reactions by a female when chased or mounted by a male.

Data analysis

Data on the absolute numbers of melanic and succinic phenotypes observed in the field were analysed by factorial ANOVA (SPSS, 1998), followed by one-way ANOVA to compare spring versus autumn at all four locations in both years. A paired *t*-test was used to compare the numbers of each phenotype observed in spring and fall in each year. Data from the mating experiments were analysed by factorial ANOVA with 'generation', 'male phenotype' and 'female phenotype' as independent fixed factors, followed by one-way ANOVA of individual factors within and between generations. Treatment means were separated by LSD ($\alpha = 0.05$) when more than two groups were compared.

Results

Seasonal variation in Harmonia axyridis phenotypes

A total of 205 996 *H. axyridis* adults were recorded over the 2 years of observation, of which 38.2% were melanic and 61.8% succinic. As melanism in *H. axyridis* is controlled by a dominant allele at a single locus (Ueno *et al.*, 1998), the equilibrium frequency of the melanic allele in the population was calculated to be $1.0–0.618^{-2} = 0.214$.

There was no effect of the random factor year ($F_{(1,79)} = 0.103$, $P = 0.749$), so data were pooled for both years and a three-way ANOVA was run with 'season', 'date', and 'location' as fixed factors (Table 1). There were significant main effects of season and location on the observed proportion of melanic beetles, and the 'season × location' interaction was also significant. The proportion of melanic beetles was significantly greater in spring samples than in fall samples at all four locations in both years (Fig. 1). There was no significant difference in absolute abundance of the two colour forms in the spring of 2006 ($t = 1.812$, d.f. $= 19$, $P = 0.086$) or 2007 ($t = 1.919$, d.f. $= 19$, $P = 0.070$), but succinics outnumbered melanics in autumn in 2006 ($t = 5.201$, d.f. $= 19$, $P < 0.001$) and 2007 ($t = 5.304$, d.f. $= 19$, $P < 0.001$).

Choice mating test

The time to copula in the choice test was strongly affected by 'generation', 'female phenotype', and 'male phenotype', and the two-way and three-way interactions were all significant (Table 2). Duration of copula was affected only by generation, and the interaction between male and female phenotypes was also significant. Egg retention time was strongly affected by female phenotype, with significant 'generation × female phenotype' and 'generation × male phenotype' interactions. Given the interactions between phenotypes, and between generation and phenotype, we compared main effects between generations, and then analysed spring and autumn generations separately.

Melanic pairs took significantly longer to form in the spring generation than the other three phenotype combinations ($F_{3,36} = 237.40$, $P < 0.001$), the latter forming on average in similar times (Fig. 2a). There was also a difference among phenotype combinations in the duration of copula ($F_{3,36} = 8.47$, $P < 0.001$), with copula lasting longer in dissimilar pairs than in similar ones (Fig. 2b). There were significant differences among phenotypes with respect to the egg retention time of spring females ($F_{3,36} = 7.91$, $P < 0.001$). Melanic females delayed oviposition

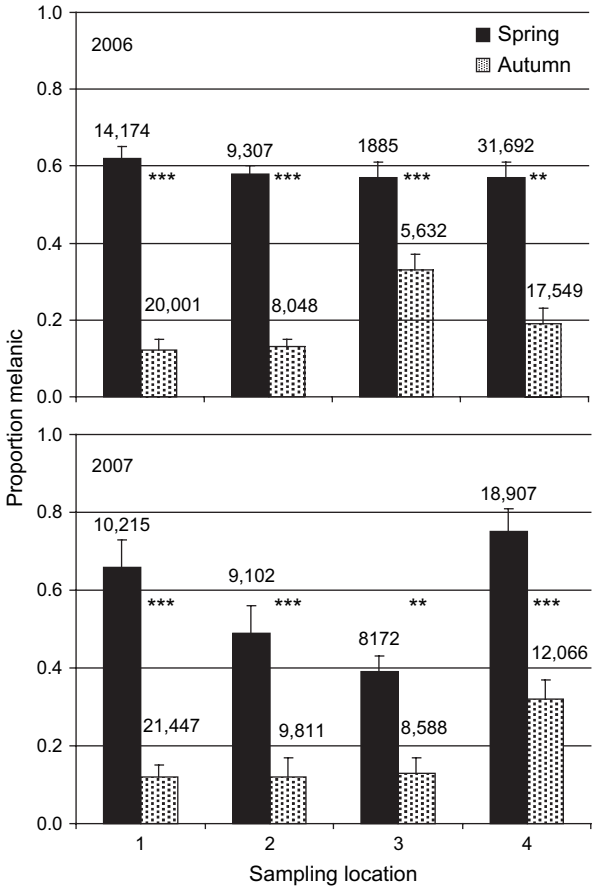

Fig. 1. Proportions of melanic (vs succinic) *Harmonia axyridis* observed at four sampling locations around Beijing, China. Each bar depicts the mean (+SEM) of five sampling dates in either spring or autumn in each of the 2 years. Numbers above bars represent total numbers of beetles observed. Asterisks indicate significant differences between absolute numbers at each site (one-way ANOVA, ***, $\alpha \leq 0.001$, **, $\alpha \leq 0.01$). 215 × 279 mm (600 × 600 DPI).

Table 1. Factorial ANOVA of fixed factors affecting the proportion of melanic *Harmonia axyridis* adults observed around Beijing, China in 2 years.

		Proportion melanic	
Source of variation	d.f.	*F*	*P*
Season	1	171.00	<0.001
Date	4	0.49	0.745
Location	3	3.23	0.032
Season × date	4	1.85	0.138
Season × location	3	3.30	0.030
Date × location	12	0.62	0.815
Season × date× location	12	0.87	0.581
Error	40		

Table 2. Factorial ANOVA of fixed factors affecting the behaviour of virgin *Harmonia axyridis* adults of spring and summer generations in a choice mating test with 0.5 sex ratio and equal numbers of melanic and succinic males and females. Mating pairs were isolated as they formed and phenotype frequencies in the arena were held constant by continuous replacement.

		Time to copula		Duration of copula		Egg Retention Time	
Source of variation	d.f.	*F*	*P*	*F*	*P*	*F*	*P*
Generation	1	31.99	<0.001	32.76	<0.001	0.84	0.364
♀ phenotype	1	61.71	<0.001	1.28	0.262	28.06	<0.001
♂ phenotype	1	60.08	<0.001	2.20	0.143	3.25	0.076
Generation × ♀	1	335.59	<0.001	1.15	0.288	33.38	<0.001
Generation × ♂	1	232.33	<0.001	2.92	0.092	8.04	0.006
♀ × ♂	1	150.18	<0.001	16.23	<0.001	3.10	0.082
Generation × ♀ × ♂	1	162.83	<0.001	0.04	0.836	5.03	0.028
Error	72						

longer than succinic females when mated by melanic males, but were the fastest to lay when mated by a succinic male. Alternately, succinic females had an intermediate egg retention time that did not vary with the phenotype of their mate (Fig. 2c). Melanic males took almost four times as long to obtain a mate in the spring generation as did succinic males ($F_{(1,38)} = 14.31$, $P = 0.001$) and subsequent egg retention time by their females averaged 1.5 h longer ($F_{(1,38)} = 11.11$, $P = 0.002$).

Adults of the autumn generation generally showed opposite behavioural trends. Melanic pairs were the fastest to form and succinic pairs the slowest, with mixed pairs intermediate and not significantly different from one another ($F_{3,36} = 39.02$, $P < 0.001$; Fig. 2d). There was also variation among pair types in the duration of copula ($F_{3,36} = 3.21$, $P = 0.037$), melanic females being abandoned earlier by melanic males than by succinic males, but no effect of male phenotype on

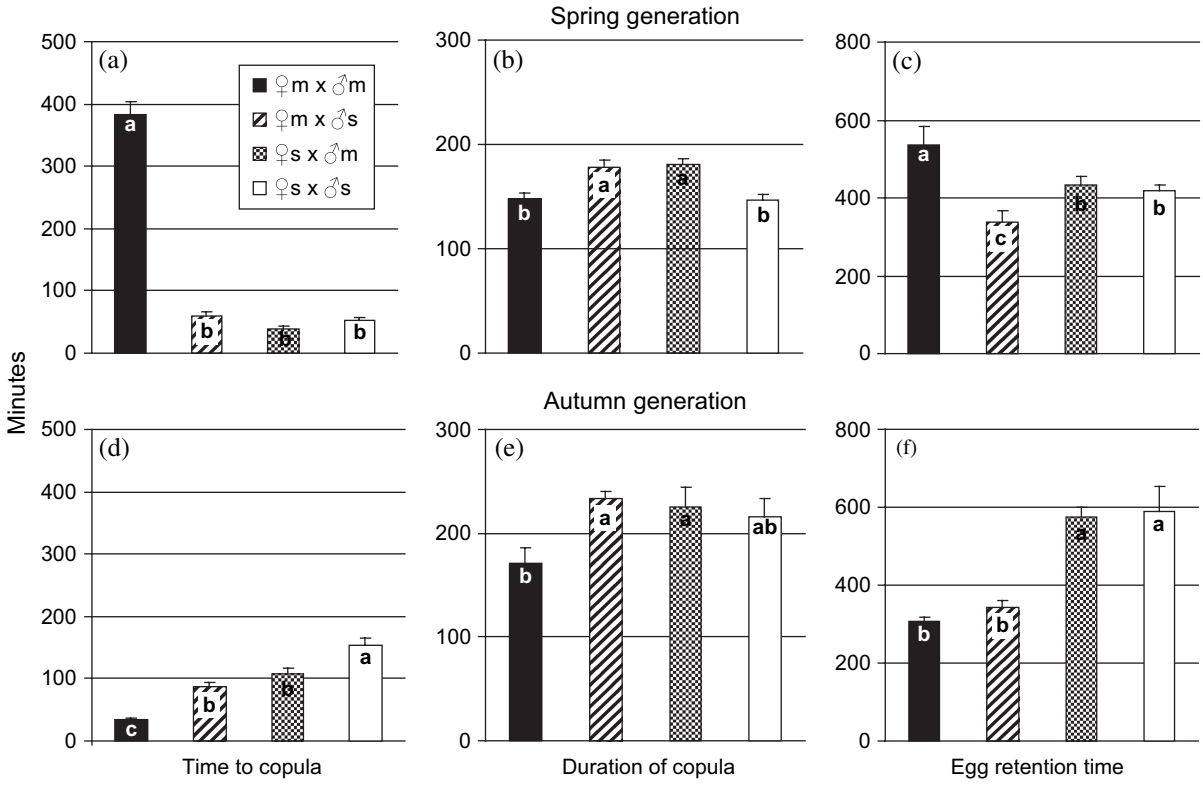

Fig. 2. Mean (+SEM) time to copula, duration of copula, and female egg retention time for various *Harmonia axyridis* phenotype combinations in choice mating tests (melanic ♀ + melanic ♂, solid columns; melanic ♀ + succinic ♂, hatched columns; succinic ♀ + melanic ♂, shaded columns; succinic♀ + ♂ succinic, open columns). Upper figures (a–c) represent data from the spring generation and lower figures (d–f), data from the autumn generation. Columns bearing the same letter were not significantly different (LSD, $\alpha = 0.05$). 253 × 189 mm (600 × 600 DPI).

the mating duration of succinic females (Fig. 2e). Melanic females displayed shorter egg retention times than succinic females ($F_{3,36} = 17.16$, $P < 0.001$; Fig. 2f). Succinic males took almost three times as long to obtain a mate as did melanic males ($F_{(1,38)} = 13.27$, $P = 0.001$), but there was no difference in the subsequent egg retention time of their females ($F_{(1,38)} = 0.19$, $P = 0.662$).

Melanic pairs took more than ten times as long to form in the spring generation as in the autumn ($F_{(1,18)} = 300.74$, $P < 0.001$) and females from these pairs had much longer egg retention times ($F_{(1,18)} = 25.69$, $P < 0.003$). However, there was no difference in duration of copula ($F_{(1,18)} = 2.10$, $P = 0.165$). In contrast, succinic pairs formed three times as quickly in the spring generation as in the autumn ($F_{(1,18)} = 65.32$, $P < 0.001$) and had significantly shorter egg retentions times ($F_{(1,18)} = 6.66$, $P = 0.019$), although they coupled for shorter periods ($F_{(1,18)} = 13.80$, $P = 0.002$). Mixed pairs took significantly longer to form in the autumn generation than in the spring generation ($♀_m + ♂_s$: $F_{(1,18)} = 12.02$, $P = 0.003$; $♀_s + ♂_m$: $F_{(1,18)} = 50.39$, $P < 0.001$) but coupled for longer periods ($F_{(1,18)} = 29.85$, $P < 0.001$ and $F_{(1,18)} = 5.87$, $P = 0.026$, respectively). Succinic females mated by melanic males had longer egg retention times in the autumn than in the spring ($F_{(1,18)} = 9.65$, $P = 0.006$), but melanic females mated by succinic males did not differ between seasons ($F_{(1,18)} = 0.21$, $P = 0.886$).

No-choice mating test

All fixed factors had a significant effect on time to copula in the no-choice experiment, and all two-way interaction terms were significant, although the three-way interaction term was not (Table 3). Duration of copula was affected only by generation, but the egg retention time was affected by all three factors and there were significant 'generation × female phenotype', 'generation × male phenotype', and 'generation × female phenotype × male phenotype' interactions.

When the various pair combinations were compared separately for each generation, the patterns largely paralleled those obtained in the choice experiment. Adults of the spring generation varied in time to onset of copulation depending on the phenotypes combined ($F_{3,36} = 71.96$, $P < 0.001$), the combination $♀_m + ♂_m$ taking significantly longer to couple than the others that did not differ among each other (Fig. 3a). There was no effect of phenotype combination on the duration of copula ($F_{3,36} = 1.02$, $P = 0.396$, Fig. 3b). However, spring females from different pairings varied in their egg retention period ($F_{3,36} = 11.41$, $P < 0.001$) with melanic females, as a group, delaying oviposition longer than succinic females ($F_{1,38} = 22.44$, $P < 0.001$) and no consistent effect of male phenotype ($F_{1,38} = 0.068$, $P < 0.001$; Fig. 3c).

Beetles of the autumn generation once again exhibited the reverse pattern of the spring generation. Time to onset of copula varied according to phenotype combination ($F_{3,36} = 34.72$, $P < 0.001$), with melanic pairs forming the fastest, succinics the slowest, and mixed pairs in an intermediate period (Fig. 3d). There was no significant variation in duration of copula among pair types ($F_{3,36} = 1.02$, $P = 0.396$, Fig. 3e), but females varied in their egg retention time according to phenotype combination ($F_{3,36} = 44.77$, $P < 0.001$). Melanic females laid earlier than succinic females, and both phenotypes laid earlier when mated by a melanic male than when mated by a succinic male (Fig. 3f).

Comparing generations, melanic pairs took four times as long to form in the spring generation as in the autumn ($F_{1,18} = 67.35$, $P < 0.001$), coupled for shorter periods ($F_{1,18} = 17.82$, $P = 0.001$), and had shorter egg retention by females ($F_{1,18} = 7.12$, $P = 0.016$). In contrast, succinic pairs took six times as long to form in the autumn generation as in the spring ($F_{1,18} = 109.03$, $P < 0.001$), coupled for shorter periods ($F_{1,18} = 9.73$, $P = 0.005$), and held their eggs for six times as long ($F_{1,18} = 107.23$, $P < 0.001$). Mixed pairs again took longer to form in the autumn than in the spring ($♀_m + ♂_s$: $F_{1,18} = 13.12$, $P = 0.002$; $♀_s + ♂_m$: $F_{1,18} = 39.58$, $P < 0.001$). There was no effect of generation on the mating duration of succinic males with melanic females ($F_{1,18} = 1.88$, $P = 0.187$), but melanic males abandoned succinic females earlier in the spring generation than in the autumn ($F_{1,18} = 14.81$, $P = 0.001$). Autumn females from mixed pairs retained eggs significantly longer than did their counterparts in the spring generation ($♀_m + ♂_s$, $F_{1,18} = 84.81$, $P < 0.001$; $♀_s + ♂_m$ $F_{1,18} = 238.69$, $P < 0.001$).

Female rejection behaviour

Videotaped observations of the mating experiments revealed that females were able to influence male mating success. Male

Table. 3. Factorial ANOVA of fixed factors affecting the behaviour of virgin *Harmonia axyridis* adults of spring and summer generations in a no-choice mating test with melanic and succinic phenotypes arbitrarily assigned to different pair-wise combinations.

		Time to copula		Duration of copula		Egg retention time	
Source of variation	d.f.	*F*	*P*	*F*	*P*	*F*	*P*
Generation	1	76.85	<0.001	21.53	<0.001	283.21	<0.001
♀ phenotype	1	11.82	0.001	1.57	0.214	69.27	<0.001
♂ phenotype	1	14.38	<0.001	0.48	0.492	30.76	<0.001
Generation × ♀ phenotype	1	102.53	<0.001	0.08	0.773	119.28	<0.001
Generation × ♂ phenotype	1	80.51	<0.001	0.00	0.960	28.79	<0.001
♀ × ♂ phenotype	1	24.58	<0.001	0.14	0.707	1.17	0.282
Generation × ♀ × ♂ phenotype	1	0.07	0.795	0.19	0.668	6.38	0.015
Error	72						

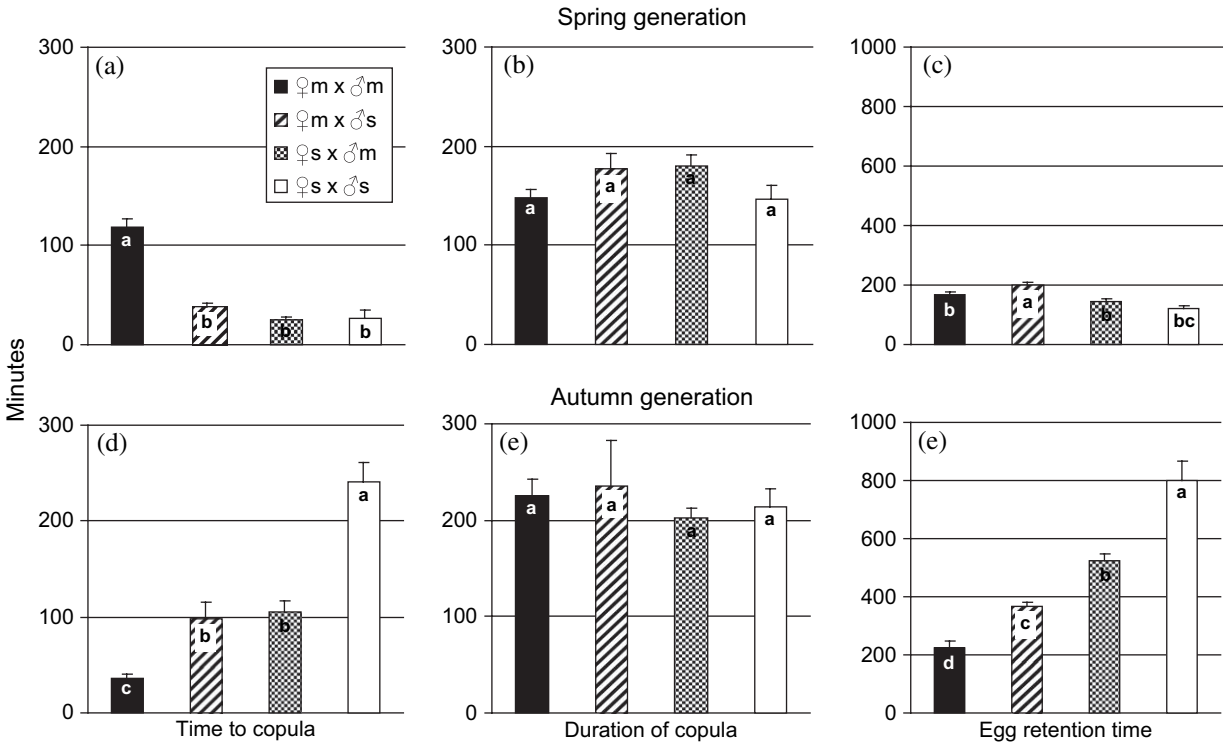

Fig. 3. Mean (+SEM) time to copula, duration of copula, and female egg retention time for various *Harmonia axyridis* phenotype combinations in no-choice mating tests (melanic ♀ + melanic ♂, solid columns; melanic ♀ + succinic ♂, hatched columns; succinic ♀ + melanic ♂, shaded columns; succinic ♀ + succinic ♂, open columns). Upper figures (a–c) represent data from the spring generation and lower figures (d–f), data from the autumn generation. Columns bearing the same letter were not significantly different (LSD, $\alpha = 0.05$). 253 × 189 mm (600 × 600 DPI).

advances were rebuffed through avoidance (the female actively walking or running away), passive resistance (abdominal retraction to prevent genital contact), or active displacement (the female shaking her abdomen to dislodge the male).

The relative frequencies of rejection events among phenotype combinations mirrored closely the variation observed in time to copula; this was true in both generations and for both choice and no-choice tests. There was a significant effect of generation on the number of rejection events in both the choice ($F_{(1,72)} = 6.61$, $P = 0.010$) and no-choice ($F_{(1,72)} = 77.84$, $P < 0.001$) tests and the interactions 'generation × male phenotype' (choice: $F_{(1,72)}$ 100.03, $P < 0.001$; no-choice: $F_{(1,72)} = 141.86$, $P < 0.001$) and 'generation × female phenotype' (choice: $F_{(1,72)} = 124.35$, $P < 0.001$; no-choice: $F_{(1,72)} = 141.86$, $P < 0.001$) were also significant, so spring and autumn generations were analysed separately.

Melanic females discriminated strongly against melanic males in the spring generation, but succinic females did not (choice: $F_{3,36} = 45.72$, $P < 0.001$; Fig. 4a; no-choice: $F_{3,36} = 56.72$, $P < 0.001$; Fig. 5a). In the autumn generation, melanic and succinic females both rejected succinic males more often than they did melanic males in the choice test ($F_{3,36} = 43.65$, $P < 0.001$; Fig. 4b) and the no-choice test ($F_{3,36} = 54.47$, $P < 0.001$; Fig. 5b).

In the choice test, melanic females were less receptive in spring than in autumn ($F_{(1,38)} = 26.64$, $P < 0.001$), whereas the reverse was true for succinic females ($F_{(1,38)} = 17.08$, $P < 0.001$). Melanic females rejected melanic males nine times more often in the spring generation than in the autumn ($F_{(1,18)} = 200.00$, $P < 0.001$) and succinic females rejected succinic males more than twice as often in the autumn as they did in the spring ($F_{(1,18)} = 115.20$, $P < 0.001$). However, rejection frequencies did not differ across generations for females in mixed pairs ($♀_m + ♂_s$: $F_{(1,18)} = 0.00$, $P = 1.000$; $♀_s + ♂_m$: $F_{(1,18)} = 0.717$, $P = 0.411$).

In the no-choice test, there was no significant difference between generations in the overall receptivity of melanic females ($F_{(1,38)} = 0.964$, $P = 0.332$), but they rejected melanic males seven times more often in the spring than in the autumn ($F_{(1,18)} = 175.94$, $P < 0.001$) and succinic males four times more often in the autumn than in the spring. ($F_{(1,18)} = 50.78$, $P < 0.001$). In contrast, succinic females rejected males more often in the autumn than in the spring regardless of their phenotype ($F_{(1,38)} = 114.99$, $P < 0.001$).

Discussion

Succinic *H. axyridis* were more common on average than melanics in habitats around Beijing, and out-numbered them five to one in autumn generations (Fig. 1). However, the relative abundance of melanics increased markedly in spring generations, such that both phenotypes were observed at similar frequencies. Similarly, Osawa and Nishida (1992) observed a Japanese population of *H. axyridis* in which succinics

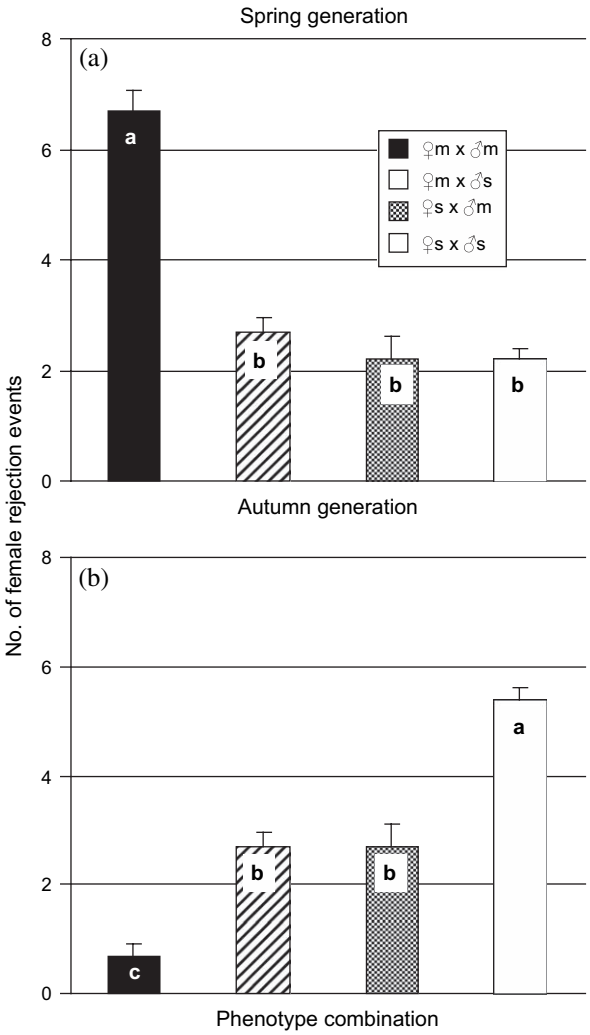

Fig. 4. Mean (+SEM) numbers of mate rejection events for *Harmonia axyridis* females in various phenotype combinations in choice mating tests (melanic ♀ + melanic ♂, solid columns; melanic ♀ + succinic ♂, hatched columns; succinic ♀ +melanic ♂, shaded columns; succinic ♀ + ♂ succinic, open columns). Upper figure (a) represents data from the spring generation and lower figure (b), data from the autumn generation. Columns bearing the same letter were not significantly different (LSD, $\alpha = 0.05$). 189 × 253 mm (600 × 600 DPI).

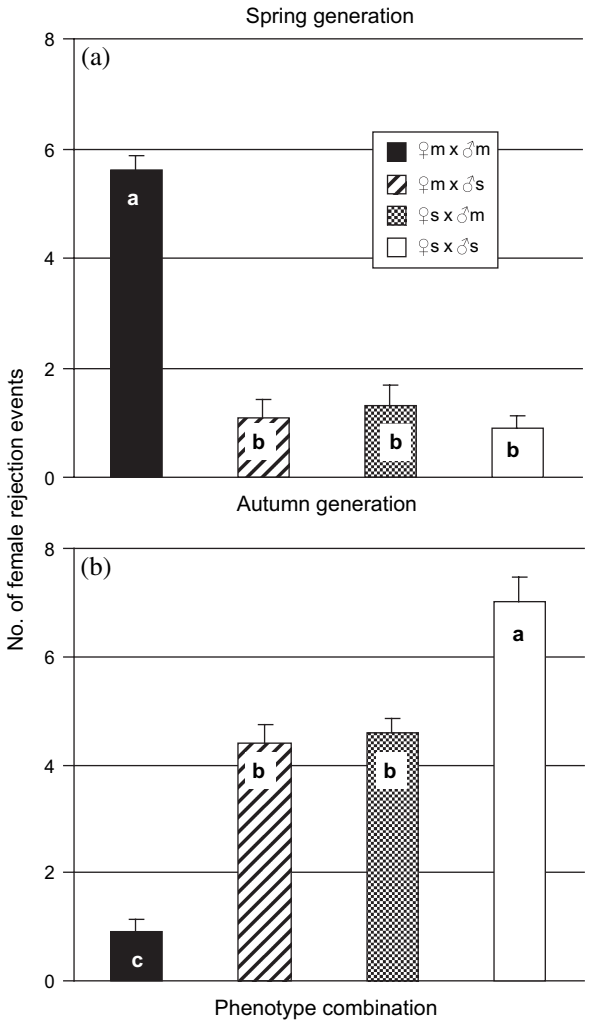

Fig. 5. Mean (+SEM) numbers of mate rejection events for *Harmonia axyridis* females in various phenotype combinations in no-choice mating tests (melanic ♀ + melanic ♂, solid columns; melanic ♀ + succinic ♂, hatched columns; succinic ♀ + melanic ♂, shaded columns; succinic ♀+ succinic ♂, open columns). Upper figure (a) represents data from the spring generation and lower figure (b), data from the autumn generation. Columns bearing the same letter were not significantly different (LSD, $\alpha = 0.05$). 189 × 253 mm (600 × 600 DPI).

increased in relative abundance during summer via assortative mating, although melanic forms predominated in that population. Seasonal cycles of melanism occur in coccinellids and other insects (e.g. Rodriguez del Bosque, 2004) and tend to be observed in indigenous populations that normally have two generations per year, e.g. *C. septempunctata* in India (Rhamhalinghan, 1988) and *H. axyridis* in China (Tan, 1949) and Japan (Osawa & Nishida, 1992).

The shift towards increasing abundance of melanic forms in spring is consistent with the theory of thermal melanism (Muggleton *et al.*, 1975a; Muggleton, 1978; Brakefield & Wilmer, 1985). For example, Muggleton *et al.* (1975a) showed that the frequency of melanic *A. bipunctata* in Britain was negatively correlated with the number of hours of bright sunlight. Brakefield (1984b) showed that overwintering melanic *A. bipunctata* emerged earlier, became active sooner, dispersed further, and reproduced earlier in spring than did succinic forms. Similarly, Soares *et al.* (2003) demonstrated a lower thermal optimum for prey consumption in a melanic versus a succinic morph of *H. axyridis*, that would presumably extend diurnal foraging periods when temperatures are cool. However, in hot summer weather, melanic forms become disadvantaged relative to succinics, due to their lower cuticular reflectance and greater temperature excess (Brakefield & Wilmer, 1985; Stewart & Dixon, 1989; de Jong *et al.*, 1996). Thus, various consequences

of the phenotypes for survival, development, dispersal or reproduction could select for the observed seasonal cycles of melanism in these *H. axyridis* populations, with environmentally cued mate preference criteria providing the proximal mechanism for phenotype shifts.

There was no difference between generations in the overall speed of pair formation in the choice test, but in the no-choice test, pairs formed more than twice as quickly in the autumn generation as in the spring, independent of phenotype, and females laid eggs almost three times as quickly (Fig. 3). Given the effectiveness of female rejection behaviour (Obata, 1988) and its excellent correspondence to data for time to copula in this study, we conclude that intrinsic differences in female receptivity are largely responsible for the differences between generations in time to copula in the no-choice test. Although female receptivity is linked to nutritional status (Obata, 1988) and females are able to resorb oocytes when food becomes scarce (Osawa, 2005), such effects were absent from our study as a result of the *ad libitum* food supply. As both spring and autumn females presumably defer oviposition for some period (as a result of hibernation and aestivation, respectively), it is not clear why autumn females should be initially less receptive to an arbitrarily assigned male than their spring counterparts, and why they retain their eggs for longer periods before utilizing the sperm of less-preferred males. However, the lower receptivity of succinic females in choice tests, their longer times to copula, and longer egg retention periods in autumn compared with spring, are all traits consistent with their inferred status as the phenotype more tuned to summer/autumn conditions.

Generation had a large and consistent effect on the duration of copula in both the choice and no-choice tests, with pairs coupling for longer periods in the autumn generation than in the spring. As termination of copula, which is a form of female rejection, normally occurs within 2 min of genital contact or less (Osawa, 1994), we conclude that durations of the prolonged copulations observed in these experiments were under male control, as concluded by Srivastava and Omkar (2004) for *C. septempunctat*a, and thus reflect a measure of male reproductive investment. This inference was supported by the finding of no effect of male phenotype on the duration of copula in either mating test. Omkar *et al.* (2006) interrupted copulations of *Cheilomenes sexmaculata* (F.) and *Coelophora saucia* (Mulsant) to demonstrate that female fecundity and fertility both increased with duration of mating beyond the threshold required for insemination. In *H. axyridis*, it has been estimated that successful insemination requires that copula last around 2 h (Obata & Johki, 1991). Duration of copula may correlate with the amount of sperm transferred to the female or the amount of nutrition contributed to the female via the spermatophore (Obata, 1987; Obata & Hidaka, 1987). As males monopolise a female during copula, its duration could also reflect the amount of effort invested in guarding a female from other males (Simmons & Siva-Jothy, 1998). In addition, de Jong *et al.* (1998) found that paternity improved with duration of copula for *A. bipunctata* males mating with previously-mated females. Thus, it is notable that mating duration did not differ among phenotype combinations in the no-choice experiment, but did in the choice experiment where the males competed with other males and encountered multiple females.

The observed increase in reproductive investment per female by autumn males, compared with spring males would be advantageous if encounter rates with females were consistently lower in summer, or if sperm competition were more intense. In *H. axyridis*, the paternity advantage of the second male (P_2) is positively correlated with the duration of the second mating (Ueno, 1994). Although a second male has an advantage in paternity over a first male (Ueno, 1996; Ueno *et al.*, 1998), the first male may reduce the margin of this advantage with a larger ejaculate load, because some amount of sperm mixing is inevitable (e.g. de Jong *et al.*, 1998). If autumn females are more likely than spring females to be previously mated, to delay oviposition, or to mate again prior to oviposition, autumn males would improve their paternity by investing more sperm in each mating.

Male phenotype had a strong effect on time to copula in both mating tests, supporting the view that female preference for males is strongly affected by their elytral colour, even though additional cues may be involved (Osawa & Nishida, 1992; Ueno *et al.*, 1998). In particular, body size (Tomlinson *et al.*, 1995) but also possibly the semiochemicals involved with sex recognition (Hemptinne & Dixon, 2000). Male phenotype did not affect egg retention time when females were allowed to select males (with the exception of melanic females in spring), but strongly affected it when they were not (Fig. 3). Thus females may accept less-preferred males in the absence of alternatives, but wait longer before utilizing their sperm, ostensibly to increase their chances of finding a more preferred male phenotype. The ability of *H. axyridis* females to manipulate paternity post-copula via delayed oviposition and secondary copulations is an example of 'cryptic female choice' (Eberhard, 1996).

The patterns of variation in female rejection behaviour reveal that females express generational cycles of preference for male phenotypes, and are able to directly influence their mating success. The strength of correspondence between female rejections of male phenotypes and their time to copula, supports the interpretation that pair formation is largely under female control. Whereas our data do not indicate any effect of female phenotype on female mating success, males provided longer copulations to females of dissimilar than similar phenotype in the choice test, suggesting that reproductive investment by males was affected by female phenotype.

The strong effects of female phenotype on time to copula in both choice and no-choice tests indicate that the strength of preference for a male phenotype depends on the female's phenotype. Consider that succinic females in autumn will contribute no melanic offspring to the overwintering generation unless they acquire a melanic male, whereas most melanic females are heterozygotes that will still produce 50% melanic offspring when mated by a succinic male. Hence, the latter are more receptive to succinic males in autumn and do not delay oviposition as long after mating with them as do their succinic counterparts. Thus, females whose phenotype was appropriate for the subsequent generation, appeared more willing to accept males of the less preferred phenotype than females whose phenotype was

inappropriate, suggesting that the strength of female preference could be modified by genes linked to elytral colour.

The egg retention times obtained from the no-choice experiment appear to reflect levels of female satisfaction with their assigned mates. In the autumn generation, both succinic and melanic females laid earlier when mated to a melanic male, but melanic females laid faster than succinic females regardless of the phenotype of their mate. Thus, a female's egg retention behaviour was affected by her own phenotype as well as the phenotype of her mate. In the spring generation, long egg retention times by melanic females mated by melanic males are expected if females are to increase their chances of re-mating with the more preferred succinic phenotype. However, it is not clear why melanic females should retain eggs for so long when mated by the (preferred) succinic males in spring, nor why succinic females should oviposit almost as quickly when mated by melanic males as when mated by succinics. It is possible that seasonal shifts in egg retention behaviour linked to female phenotype, override any effects of male phenotype on this particular trait. Similarly, the lower overall receptivity of melanic females in spring and of succinic females in autumn, evident from the rejection data, are phenomena difficult to explain in terms of individual selection. Although the expression of lower receptivity in females of less preferred phenotype would serve to reinforce the observed cycles of phenotype abundance, assigning adaptive significance to such an effect would require an argument for group selection.

True (2003) reviewed the molecular processes of melanization in insects and described mechanisms whereby melanism may have pleiotropic effects on other traits. It is thus possible that alleles for melanism exerted pleiotropic effects on genes affecting female receptivity, egg retention time, and male mating investment in our experiments. However, the observed effects of generation occurred independent of phenotype and occurred despite standardised rearing conditions, indicating that they were not directly contingent on environmental signals. Nevertheless, it was clear that effects of generation caused shifts in behaviour adaptive for seasonal environments, raising the possibility that maternal environmental conditions may have epigenetic effects on the expression of reproductive traits in progeny via selective penetrance. Subsequent work will address the possibility of maternally-mediated epigenetic effects. Alternatively, multiple linkage disequilibria may exist among various 'season-specific' alleles, that cause them to alternate in frequency between generations in tandem with alleles controlling elytral colour and female preference. Such linkages would be tenuous at best and, in the absence of stabilising selection, rapidly dismantled by independent assortment, leading to the disappearance of cyclical phenomena and shifts in the frequencies of *H. axyridis* phenotypes to new equilibria. Either explanation would be consistent with the rapid divergence of phenotype frequencies observed when populations are reared for successive generations under constant laboratory conditions (e.g. Seo *et al.*, 2008), or when the species becomes invasive in novel geographic regions (e.g. Berkvens *et al.*, 2007). Future research is warranted to clarify the relative roles of genetic and epigenetic factors in generating the seasonal cycles of behaviour observed in these and other polymorphic insect populations.

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