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Enriching early adult environment affects the copulation behaviour of a tephritid fly

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SUMMARY

Early adult experiences in enriched environments favours animal brain and behavioural development ultimately resulting in an increased fitness. However, measuring the effect of environmental enrichment in animal behaviour in nature is often a complicated task, considering the complexity of the natural environment. We expanded previous studies to evaluate how early experience in an enriched environment affects copulation behaviour when animals are confronted with a complex semi-natural environment. *Anastrepha ludens* flies are an ideal model system for studying these effects because their natural habitats differ significantly from the cage environments in which these flies are reared for biological control purposes. For example, in the field, males form leks of up to six individuals. Each male defends a territory represented by a tree leaf whereas in rearing cages, territories are completely reduced because of the high population density. In a series of three experiments, we observed that male density represented the most influential stimulus for *A. ludens* male copulation success. Males that experienced lower densities in early adulthood obtained the highest proportion of copulations. By contrast, female copulation behaviour was not altered by female density. However, exposure to natural or artificial leaves in cages in which flies were kept until tested influenced female copulation behaviour. Females that were exposed to enriched environments exhibited a shorter latency to mate and shorter copulation durations with males than females reared in poor environments. We discuss the influence of early experience on male copulation success and female-mating choosiness.

Key words: *Anastrepha*, Tephritidae, early adult experience, environmental enrichment, copulation behaviour, population density

INTRODUCTION

The standard definition of an enriched environment is ‘a combination of complex inanimate and social stimulation’ (Rosenzweig et al., 1978). The objective of environmental enrichment is to provide those stimuli that promote the expression of species-appropriate behavioural and mental activities (van Praag et al., 2000). In laboratories and zoos, environmental modifications typically consist of enhanced social interactions and stimulation of exploratory and motor behaviour with objects, such as toys, ladders, tunnels and a running wheel for voluntary physical exercise (van Praag et al., 2000). In the case of genetic conservation programmes, the aim of environmental enrichment is to maximise similarities between the captive environment and the environment where the animals are destined to be released (Newberry, 1995). In this situation, animals may be exposed to natural food items or predators to increase the range of experiences acquired by each individual, facilitate learning of characteristic cues associated with each stimulus and to develop behavioural flexibility in response to a dynamic environment. The aim of such practices is ultimately to improve each animal’s likelihood of survival and reproduction once released into the wild (Newberry, 1995).

Enriching captive rearing environments clearly influences, in a positive way, physiological and behavioural ontogeny (Kolb and Whishaw, 1998; van Praag et al., 2000). For example, the simple manipulation of adding stones to a standard rearing tank can dramatically alter the growth of specific brain structures in steelhead trout alevins [*Oncorhynchus mykiss* (Kihslinger and Nevitt, 2006)]. Exposing animals to an enriched environment at an early age favours behavioural flexibility to different situations and also reduces the development of behavioural tendencies such as boldness and aggression that could be detrimental to the survival and reproduction of individuals (Kelley et al., 2005; Kelley et al., 2006; Salvanes et al., 2007). Also, social experiences during pre-reproductive life stages may exert a strong influence on adult reproductive behaviour (Hebets, 2003).

In insects, adult experience has been linked to changes in the volume of the mushroom bodies – an organ implicated in olfactory and visual learning and memory (Sivinski, 1989a; deBelle and Heisenberg, 1994; Heisenberg et al., 1995; Fahrbach et al., 1998). Stimulating events in early development (i.e. in the egg or larval stages and during adulthood) can have long-lasting effects on behaviour because of an irreversible developmental effect. For instance, in *Drosophila melanogaster*, development of the optic lobe is affected by the light regimes in which the animals were reared as adults, resulting in differences in mating behaviour (Barth et al., 1997b). Males reared under a normal light/dark cycle exhibit a mating advantage over conspecifics reared in constant darkness when competing for females reared under a normal photoperiod (Hirsch and Tompkins, 1994; Hirsch et al., 1995). Additionally, illumination regimes result in assortative mating as the latency prior to copulation is shorter with male and female pairs reared under similar photoperiodic cycles (Barth et al., 1997a). Social context and photoperiod affect chemical communication and circadian time (Levine et al., 2002; Kent et al., 2008; Krupp et al., 2008). Other conditions such as *per capita* space can also provide fitness gains. For example, male drosophilid flies reared in cages with a greater *per capita* space, exhibited a clear mating advantage over males from smaller cages (Dukas and Mooers, 2003).

In the case of tephritid fruit flies, it has been reported that contact with fruit or leaves of the host plant increases egg load and reduces copula duration in females (Alonso-Pimentel et al., 1998; Alonso-Pimentel and Papaj, 1999; Papaj, 2000; Carsten and Papaj, 2005). Also, those males that have contact with the foliage, fruit or bark of orange or guava trees are sexually more competitive than those males exposed to orange or guava odours or to the foliage of fiddlewood trees and apples (Papadoupolos et al., 2001; Shelly and Villalobos, 2004; Shelly et al., 2004). However, exposing flies to guava branches during colonisation did not improve the flies' ability to maintain their wild behavioural repertoire after five generations of laboratory culture (Leppla et al., 1983).

To date, studies reporting brain and behavioural advantages of insects kept in enriched environments have been performed under laboratory conditions using different levels of social interactions and inanimate objects. Little is known about the effects of a combination of different social environments and natural stimuli on insect behaviour. Specifically, there is a paucity of information concerning the relationship between the experience of the flies in enriched environments and their mating success in nature. To answer this question, we performed a series of experiments using as a model the tephritid fly *Anastrepha ludens* Loew. This fly is particularly well suited for studying environmental enrichment because it exhibits a rich and plastic behavioural repertoire and is fairly long-lived compared with other tephritid flies (Aluja et al., 2000; Aluja et al., 2008). Furthermore, given its pest status, it is mass-reared, irradiated and released as part of control efforts involving the sterile insect technique (SIT). The effectiveness of SIT is based on the ability of sterile mass-reared males to compete successfully with wild males for mating with wild females (Knipling, 1955; Rull et al., 2007).

In the present study, we were interested in determining: (1) whether environmental enrichment increases the copulation success of *A. ludens* males and, (2) which one of the enrichment factors tested influenced copulation success. In addition, we were interested in ascertaining if the housing conditions under which the flies emerged and reached sexual maturity influenced female mate choice. Distinguishing between these variables may also provide insights into the mechanisms underlying mating decisions in lekking tephritid.

MATERIALS AND METHODS

Study system

Anastrepha ludens flies exhibit a lek mating system, defined as non-resource based mating aggregations (Emlen and Oring, 1977), which occurs mainly at dusk (Aluja et al., 2000). In lekking tephritids, leaves of trees represent male territories that are intensely defended; each male in a lek occupies a separate leaf (Prokopy and Hendrichs, 1979; Aluja et al., 1983; Robacker et al., 1991). However, fly territories are not fixed because males move from one leaf to another according to the position of the sun (Kaspi and Yuval, 1999a; Kaspi and Yuval, 1999b). From these leaves, males produce a complex array of olfactory, auditory and visual signals to attract females to the calling arena (i.e. lek). Females choose their mates based on some poorly understood male attributes that signal their genetic or physical qualities (Sivinski, 1989b; Whittier et al., 1992). Additionally, it has been reported that oogenesis of *A. ludens* females is affected by volatiles emanated from fruit and by the sexual pheromones emitted by males (Aluja et al., 2001). In addition, female flies are able to learn the physical and chemical characteristics of host fruits when landing on them (Robacker and Fraser, 2002; Robacker and Fraser, 2005).

Under a laboratory rearing environment, flies are maintained at a density of 140,000 individuals per cage (0.722 cm^2 fly^{-1}) during 20–22 days for egg production. The cage contains a plentiful supply of food and water, and a constant light/dark regime of 12 h:12 h is used in the rearing room (Orozco-Davila et al., 2006). Conditions for holding flies before field releases are also highly crowded. Flies are held for 5–7 days either in paper bags (20 cm in length × 10 cm width × 35–45 cm in height) with approximately 4000–8000 pupae or in plastic aerial release containers (50 l PARC boxes, Rubbermaid®, Atlanta, GA, USA) with approximately 24,000 flies (Enkerlin, 2007). In addition, high-density conditions can also be problematic for wild flies used in quality control tests. Mass-rearing facilities usually test sterile male sexual compatibility and competitiveness with wild flies under field cage conditions. However, both wild and laboratory flies used for these quality control tests were kept in small cages at a high density from emergence to two days before testing (e.g. Taylor et al., 2001).

Study site

The study was carried out in the grounds of the Fruit Fly Ecology and Behaviour Laboratory of the MoscaMed/MoscaFrut mass-rearing facilities (Subdirección de Desarrollo de Métodos) located at Metapa de Domínguez, Chiapas, Mexico. Field tests were carried out under the canopies of mango trees that surround the MoscaMed/MoscaFrut facilities.

Insects

Only wild flies were used during the present study to guarantee that flies would exhibit the complete behavioural repertoire typical of the species (as mass-reared insects tend to lose components of their repertoire) (Cayol, 2000). All *A. ludens* specimens utilised in the experiments were reared from field-infested sour oranges (*Citrus aurantium* Linnaeus) collected across the municipality of Tapachula, Chiapas, Mexico. Pupae were allowed to emerge inside 50×50×50 cm cages of made of wood and a cotton mesh screen. Insects were kept under the following environmental conditions: temperature, 25±1°C; relative humidity, 60±10%; and a 12 h:12 h light:dark cycle with lights on at 07:00 h. Prior to field cage observations, males were marked on the thorax with a small spot of vinyl paint (Vinci, Vínci de México, S.A. de C.V., Mexico City, Mexico) to distinguish individuals during the experiment. Previous studies indicated that this type of mark does not interfere with fly sexual activity (Meza et al., 2005).

Research arena

Field tests were run in cages made of Amber Lumite that measured 3 m in diameter by 2 m in height (BioQuip Products, Gardena, CA, USA) (Chambers et al., 1983; Calkins and Webb, 1983). Ten potted citrus trees and 10 potted mango trees [36.9±2.6 cm (width; mean ± s.e.m.) and 175.5±5.1 cm (height)] were distributed in an alternating sequence around the inner perimeter (16 trees) and the centre (four trees) of each cage. Citrus trees had 140±8.5 leaves per tree whereas mango trees had 34.2±1.8 leaves per tree. Mean leaf area was 25.8±1.3 cm^2 for citrus and 91.5±4.2 cm^2 for mangos.

When flies reached 16-days-old, they were transported to the field for testing. Males of each treatment were released one hour prior to testing to allow them to establish territories (16:00 h). Females were released one hour later (17:00 h). One observer carefully examined all branches by standing on a stool to search the higher branches. The procedure during evening observations was to survey the tree between 17:00 h and 17:30 h to determine the location of the flies. During this time, flies were relatively

inactive. Beginning at 17:30 h, locations where fly activity was high were observed closely throughout the remainder of the evening, although frequent scans of the tree were also conducted. The number of mating pairs, latency until copulation and copulation duration were registered.

Statistical analysis

Small integer counts (e.g. 1–7) recorded for one of the response variables (i.e. number of copulations) were analysed using a generalised linear model [GLM; JMP v. 7 (SAS Institute, Cary, NC, USA)] with Poisson errors, a log-link function and type III significance tests (Crawley, 1993; Agresti, 1996). The validity of the model was determined by the examination of diagnostic parameters such as deviance, d.f. ratios and the patterns generated by plots of scaled deviance against fitted values (Crawley, 1993). Contrasts were used to test for differences in levels within a variable. For continuous response variables such as latency until copulation and copulation duration that complied with the assumptions of the analysis of variance (ANOVA), a two way ANOVA was used. The effect of cage was included as a random factor and least-square means *t*-tests were used to compare means [JMP v. 7 (SAS Institute)]. Assortative mating was estimated using the isolation index (ISI) (Cayol et al., 1999). The ISI compares the numbers of homotypic (within strain) matings with heterotypic (between strain) matings:

$$\mathrm{ISI} = \frac{(\mathrm{SS} + \mathrm{WW}) - (\mathrm{SW} + \mathrm{WS})}{\mathrm{SS} + \mathrm{WW} + \mathrm{SW} + \mathrm{WS}}, \quad (1)$$

where SS is homotypic sterile pairs, WW is homotypic wild pairs, SW is heterotypic sterile male–wild female pairs and WS is heterotypic wild male–sterile female pairs. ISI values range from –1 (negative assortative matings, i.e. an absolute preference for mating with the differing strain) to +1 (positive assortative matings, i.e. total isolation) (Cayol et al., 1999).

Experiment 1. Mating success of males exposed to three male density conditions in early adulthood

Two enrichment factors were tested: male density and cage decoration. At 12 h after emergence, flies were separated by sex and placed in 30×30×30 cm cages with a total internal surface area of 5400 cm^2, according to the following density treatments: 500 males, 100 males and 50 males per cage. These treatments were equivalent to a mean of 10.8 cm^2, 54 cm^2 and 108 cm^2 of surface area per fly, respectively. Additional sensory stimulation was provided inside some of the cages (cage decoration) by including two artificial branches containing six oval-shaped artificial leaves (6 cm width × 9 cm length) and three artificial fruits (5.5 cm minor diameter × 7.1 cm major diameter) [named the artificial stimulus treatment (AS)] or, alternatively, *C. aurantium* branches with a similar number of natural leaves as the artificial-leaf treatment (means ± s.e.m.; 6.31±0.22 cm width × 9.48±0.53 cm length) and three ripe oranges (6.17±0.73 cm minor diameter × 6.88±0.10 cm major diameter) [natural stimulus treatment (NS)]. Branches were removed from trees near to the laboratory and immediately placed in the cages. All branches and fruit were replaced every five days to freshen stimuli. The control treatment consisted of cages devoid of any introduced stimuli [without stimulus treatment (WoS)]. A total of nine treatments were tested (3 densities × 3 types of sensory stimulation). Five replicates per treatment of five different cohorts were completed. Females were kept in cages without additional stimuli and at a density of 40 females per cage, equivalent to 135 cm^2 per fly. Water and food [three parts sugar to one part hydrolysed protein (yeast hydrolysed enzymatic, ICN Biochemicals, Aurora, OH, USA)] were provided *ad libitum* on Petri dishes placed at the centre of all cages.

Five males of each treatment (45 individuals) and 40 females were released inside each field cage. The field cage test was replicated 25 times.

Experiment 2. Assortative mating and mating success of males exposed to enriched and un-enriched environments in early adulthood

To evaluate the effect of cage decoration on both males and females, we subjected individuals of both sexes to the cage-enrichment treatments described in experiment 1. The set-up for the experiment was similar to that of experiment 1, with the exception being that we used a single density of 80 individuals per cage instead of three different densities. This density was selected on the basis that no difference was observed between the treatments involving 50 and 100 males per cage in experiment 1. Thirteen male–female pairs from each treatment were released inside each field cage. The number of mating pairs, latency to the first copulation, copulation duration and site where the copulation took place were registered. Field cage observations were carried out 25 times.

Experiment 3. Effect of territory availability and type of natural stimuli on assortative mating and mating success

For this experiment, we used two different fly densities: 16 and 48 flies per cage. We increased the number of mango or citrus leaves inside the cages to 32 to obtain two conditions of territory availability: two leaves per fly and 0.67 leaves per fly, respectively. Three ripe 'Valencia' oranges or three ripe 'Ataulfo' mangoes (*Mangifera indica* L.) were placed in each cage. Undecorated cages were used as a control. As *A. ludens* prefer host fruits within the Rutacea (Baker et al., 1944), we hypothesised that stimuli from citrus leaves and fruit would influence the behaviour of flies more than those emanating from mango leaves and fruit. Seven pairs of flies from each treatment were released inside each field cage. Field cage observations were performed 12 times. As in the preceding experiments, the number of mating pairs, latency to the first copulation and copulation duration were registered.

RESULTS

Experiment 1. Mating success of males exposed to three male density conditions in early adulthood

A total of 225 copulations were recorded. The density of males per cage greatly influenced male mating success. Those males that had experienced densities of 100 or 50 males $cage^{-1}$ early in adulthood obtained significantly more mates than males that had experienced the highest density (500 males $cage^{-1}$). Cage decoration stimuli also affected male mating success but, in contrast to our expectations, those males exposed to natural stimuli obtained fewer copulations than males of the other two treatments (artificial stimuli and without stimuli). Additionally, the interaction between density and decoration stimuli was significant, indicating that the effects of cage decoration stimuli differed according to density (Fig. 1A; Table 1).

The latency to copulate and copulation duration did not differ statistically among the male density treatments (latency; $F_{2,422}$=0.91, P=0.41; duration; $F_{2,422}$=0.62, P=0.53). Also, no statistical differences were observed among the cage decoration treatments (latency: $F_{2,422}$=0.25, P=0.78; duration: $F_{2,422}$=0.57, P=0.56) or in the interaction between these two factors (latency: $F_{4,422}$=0.94, P=0.43; duration: $F_{4,422}$=1.3, P=0.26) (Fig. 1B,C).

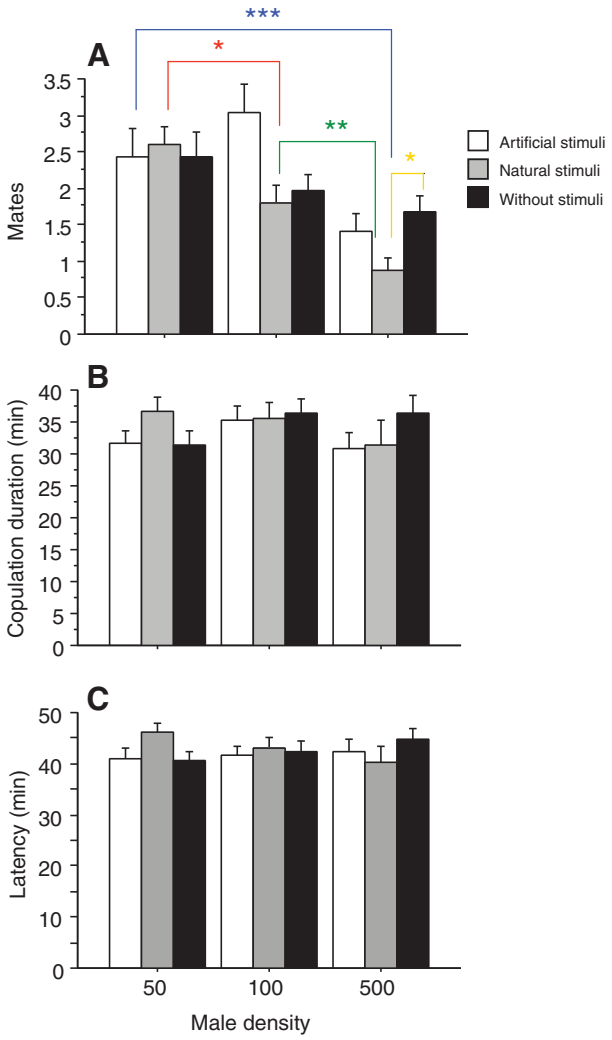


Fig. 1. Effects of rearing environment on the copulation behaviour of males that were subjected to three conspecific densities (*x* axis) and three types of decoration stimuli during the first 16 days of their life. (A) Number of mates obtained, (B) duration of copulation, and (C) latency to copulation. Bars indicate means + s.e.m. Contrasts and least-square means *t*-tests were run among treatments according to type of analysis (GLM or ANOVA) (*P<0.05; **P<0.005; ***P<0.0001).

Experiment 2. Assortative mating and mating success of males exposed to enriched and un-enriched environments in early adulthood

No significant differences were observed in the number of copulations obtained by males from any of the three environmental treatments. However, females exposed to artificial stimuli copulated less frequently than females exposed to natural or un-enriched environments (Table 2; Fig. 2A,B).

With respect to the latency to mate, females exposed to natural stimuli initiated copulation significantly earlier with heterotypic males than with homotypic males ($F_{8,491}$=2.23, P=0.025). However, these copulations were significantly shorter ($F_{8,464}$=2.05, P=0.04) (Fig. 3).

Non-assortative mating was detected because most of the ISI values were close to zero (AS:NS=–0.076; AS:WoS=0.060; NS:WoS=–0.024), indicating that individuals from the three treatments did not exhibit preferences for homo- or heterotypic males.

Table 1. Generalised linear model analysis of the number of copulations obtained by males exposed to different stimuli during early adulthood (Poisson errors, log-link)

Source	d.f.	χ^2	P
Male density	2	33.793575	**<0.0001**
Cage decoration	2	6.8221085	**0.033**
Male density × cage decoration	4	11.685449	**0.02**

Deviance=254.6626, 216 d.f. Significant values are in bold type.

Experiment 3. Effect of territory availability and type of natural stimuli on assortative mating and mating success

Neither density nor cage decoration significantly affected the number of copulations obtained by males. The interaction between both factors was also not significant. In the case of females, no statistical differences were observed according to the density. Nevertheless, the number of copulations was significantly reduced in those females that were not exposed to citrus or mango stimuli. The interaction was highly significant, indicating that the effects of the decoration varied according to female density (Table 2A,B; Fig. 4A).

Statistical differences were not detected in the copulation duration of males (density: $F_{1,135}$=0.25, P=0.61; environment: $F_{2,135}$=0.48, P=0.62; interaction: $F_{2,135}$=0.69, P=0.50) or females (density: $F_{1,135}$=0.02, P=0.89; environment: $F_{2,135}$=0.08, P=0.92; interaction: $F_{2,135}$=0.24, P=0.66) in any of the six treatments (Fig. 4B).

With respect to the latency to mate, no significant differences were detected for males (density: $F_{1,135}$=0.76, P=0.38; environment: $F_{2,135}$=0.84, P=0.43; interaction: $F_{2,135}$=0.89, P=0.41). However, females exposed to high-density conditions (48) tended to mate sooner than those under low-density conditions (16) ($F_{1,135}$=4.4, P=0.04), although no significant differences were observed among the three cage decoration treatments ($F_{2,135}$=1.15, P=0.32) and no significant interaction was observed between these factors ($F_{2,135}$=1.06, P=0.35) (Fig. 4C).

Slightly assortative mating among flies from different treatments was observed. Interestingly, disassortative matings were observed only for those flies that were not exposed to any of the environmental stimuli (Table 3; Fig. 5).

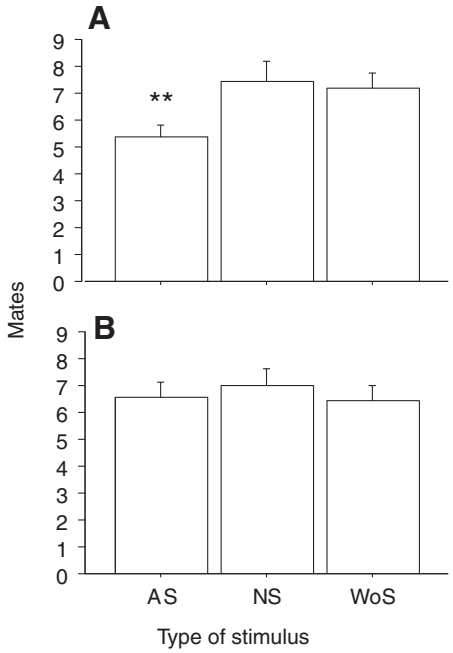
DISCUSSION

The main finding of the present study is that there are sex-specific differences in the mating behaviour of the tephritid fly *A. ludens* that experienced an enriched environment during early adulthood. Our results demonstrate that housing conditions enhanced with

Table 2. Generalised linear model analysis of the number of copulations obtained by females (A) and males (B) exposed to different stimuli during early adulthood (Poisson errors, log-link)

(A) Source	d.f.	χ^2	P
Density	1	1.2504002	0.2635
Cage decoration	2	8.4843157	**0.0144**
Density × cage decoration	2	15.01847	**0.0005**
(B) Source	**d.f.**	**χ^2**	**P**
Density	1	0.3889572	0.5328
Cage decoration	2	3.5749846	0.1674
Density × cage decoration	2	3.632383	0.1626

In A, deviance=76.0683, 66 d.f. In B, deviance=81.2817, 66 d.f. Significant values are in bold type.



Source	d.f.	χ^2	*P*
Cage decoration (females)	2	10.067486	**0.0065**
Cage decoration (males)	2	0.647675	0.7234

Deviance females = 98.063, 72 d.f. Deviance males = 101.1457, 72 d.f.
Significant values are in bold type.

Fig. 2. Number of mates (means + s.e.m.) obtained by (A) females and (B) males subjected to three different types of stimuli during the first 16 days of their life. NS=natural stimuli; AS=artificial stimuli; WoS=without stimuli. Generalised linear model (GLM) analysis of the number of copulations obtained by males and females exposed to different decoration stimuli during early adulthood (Poisson errors, log-link). A GLM contrast test was run among treatments (**P<0.01).

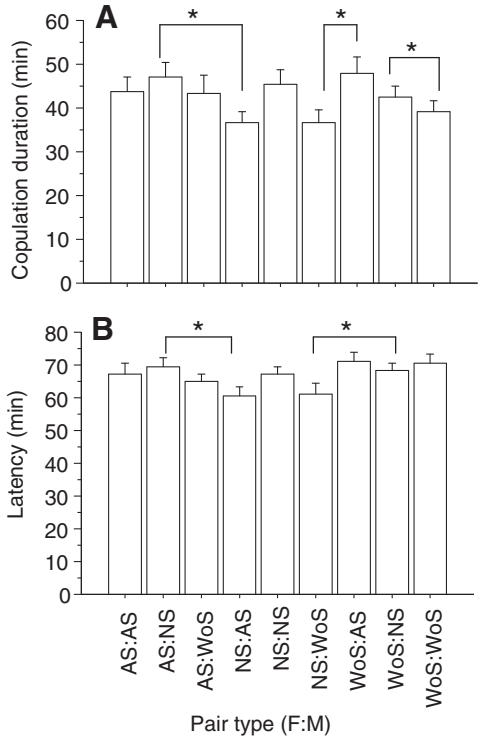


Fig. 3. Effects of rearing environment on (A) duration of copulation and (B) latency to copulation of homotypic and heterotypic pairs of flies subjected to three types of decoration stimuli the first 16 days of their life [gender order female (F):male (M)] (NS=natural stimuli; AS=artificial stimuli; WoS=without stimuli). Bars indicate means + s.e.m. A least-square means *t*-test was run among treatments (*P<0.05).

natural elements such as leaves or fruit influenced female mating behaviour but not male behaviour. In some of the treatments tested, females that had been exposed to natural stimuli exhibited a shorter latency to mate. In the case of males, a larger *per capita* area represented the most important stimulus influencing mating success, because males that experienced lower male densities in early adulthood obtained the highest proportion of copulations. However, once a minimum critical area is reached, it seems that the advantage of increasing *per capita* area reaches a plateau beyond which no additional mating advantages are accrued.

As reported for drosophilid flies, we observed that *per capita* space plays a key role in improving or maintaining certain male behavioural characteristics that increase individual mating success (Dukas and Mooers, 2003). In our present study, no significant differences were detected in mating success when comparing males kept at densities of 54 $cm^2\,fly^{-1}$ and 108 $cm^2\,fly^{-1}$ but males kept at higher densities were less successful. Apparently, a minimal critical territory or at least a minimal *per capita* area exists to maintain certain behavioural traits that confer advantages when it comes to mating. A threshold between space and social interactions may explain this pattern. It seems likely that males held at high densities spend much of their time in continuous aggressive encounters, incurring costs that affected their attractiveness. It has been argued that leks enforce high costs on males through a high rate of aggressive encounters (Gosling et al., 1987; Aspi and Hoffmann, 1998). Additionally, it has been observed that *D. melanogaster* males reduce their territoriality at high densities because no mating advantage is obtained (Hoffmann and Cacoyianni, 1990). In the case of tephritid flies, aggressive interactions, which are normally infrequent under natural conditions, only take place on leaves when males are defending territories in leks (Whittier et al., 1994; Segura et al., 2007). However, under laboratory conditions, male survival and mating success decrease significantly and male–male behavioural interactions increase significantly with increasing male density (Gaskin et al., 2002). Thus, it seems that males require a minimum amount of space to be able to exhibit their normal courtship behaviour. Alternatively, it has been demonstrated that drosophilid males produce pheromones with different compositions according to their previous social experience and the age at which social experience occurred and these changes clearly affect courtship behaviour (Siwicki et al., 2005; Svetec and Ferveur, 2005; Svetec et al., 2005a; Svetec et al., 2005b). In lekking insect species, male pheromones play a critical role for mating success. Under crowded conditions, and because of heavy competition, males may deplete the amount of pheromones present in their glands with a consequent reduction in attractiveness, as occurs in other species of flies (Jones and Hamilton, 1998; Johansson et al., 2005; Widemo and Johansson, 2006).

In lekking tephritids, males prefer to form leks on plants according to tree height, tree volume and leaf size (Whittier et al., 1992; Shelly and Whittier, 1994; Kaspi and Yuval, 1999a; Kaspi and Yuval, 1999b). Fruit fly leks are formed preferentially on specific trees whose features attract males week after week (Shelly et al., 1994).

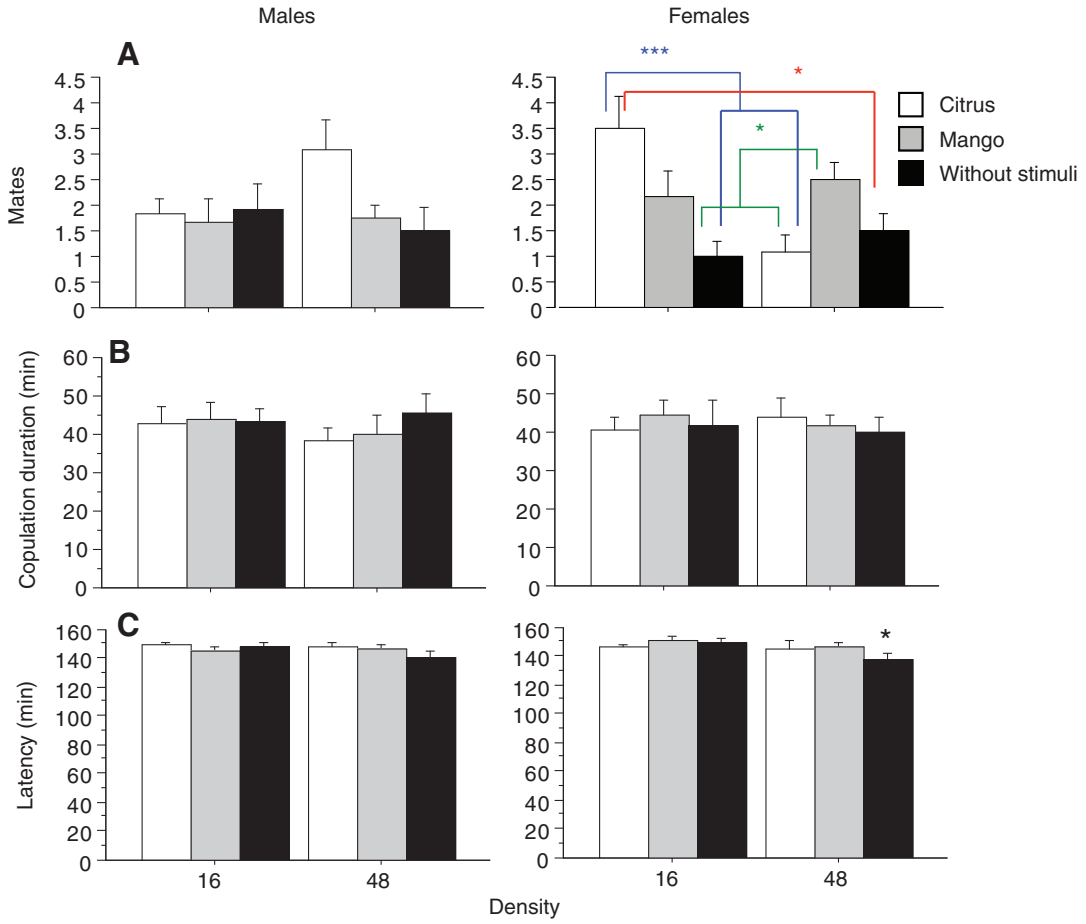


Fig. 4. Effects of rearing environment on the copulation behaviour of males (left) and females (right) that were subjected to two densities (*x* axis) and three types of stimuli during the first 16 days of their life. (A) Number of mates, (B) duration of copulation, and (C) latency to copulation. Bars indicate means + s.e.m. Contrasts and least-square means *t*-tests were run among treatments according to the type of analysis (GLM or ANOVA) (*P<0.05; ***P<0.0001).

Males move from leaf-to-leaf on these trees and no static territories are established (Whittier et al., 1992; Kaspi and Yuval, 1999a; Kaspi and Yuval, 1999b). This dynamic behaviour could explain why varying leaf availability (experiment 3) did not exert a clear influence on male mating success. However, this may indicate that allowing males to develop adequate locomotion skills and reducing the number of aggressive interactions by providing enough space for each individual is more important than physical or chemical stimuli inside the holding cage.

Submitting males to artificial or natural stimuli did not result in any mating advantage. This result is similar to that observed in *D. melanogaster* (Dukas and Mooers, 2003) but differs with the findings in another tephritid, *Ceratitis capitata* (Shelly and Villalobos, 2004; Shelly et al., 2004). In the case of *D. melanogaster*, males exposed to coloured pieces of pipe did not acquire any behavioural advantage. However, *C. capitata* males that landed on guava or orange tree leaves or fruit were more sexually successful than males that did not come into contact with those parts of the plants (Shelly et al., 2004). We suggest that, in spite of the fact that we did not observe a measurable effect of the stimulus in the case of *A. ludens* males, it could still play a role in stimulating reproduction because, for example, low numbers of homotypic WoS

Table 3. Total number of copulations according to the mating type

Males	16 Citrus	48 Citrus	16 Mango	48 Mango	16 WoS	48 WoS	**Total**
Females							
16 Citrus	8	9	7	6	9	3	**42**
48 Citrus	1	5	2	2	1	2	**13**
16 Mango	2	7	5	4	5	3	**26**
48 Mango	5	7	2	6	4	2	**26**
16 WoS	2	6	1	1	0	2	**12**
48 WoS	4	3	3	2	4	2	**18**
Total	**22**	**37**	**20**	**21**	**23**	**14**	**137**

Alphanumeric rows and columns indicate the type of stimuli that flies were exposed to. WoS=without stimuli.

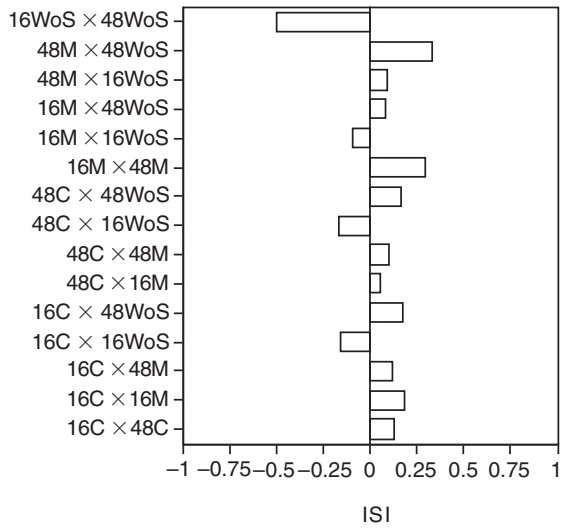


Fig. 5. Estimates of sexual isolation expressed by isolation index (ISI) coefficient values according to mating type. The ISI compares the numbers of homotypic (within strain) matings with heterotypic (between strain) matings. ISI values range from –1 (negative assortative matings, i.e. an absolute preference for mating with the differing strain) to +1 (positive assortative matings, i.e. total isolation) (Cayol et al., 1999). For example, matings between individuals of 16WoS × 48WoS combination resulted in more heterotypic (i.e. negative assortative matings) than homotypic pairs whereas in the 48M × 48WoS combination more homotypic (i.e. positive assortative matings) than heterotypic pairs were observed. WoS=without stimuli; M=mango; C=citrus.

mating pairs were observed during the present study. Nevertheless, in the case of females, some effects were observed as those flies exposed to the natural stimulus (mango or citrus) exhibited differences in mating behaviour. Females exposed to natural stimuli tended to copulate more frequently and sooner than females from the artificial or control treatments. However, latency to mate was shorter only when females copulated with males exposed to artificial stimuli or with those reared in cages without stimuli compared with the latency to mate with homotypic males. Shorter latency to copulation and shorter copulation duration were observed in the case of females of the walnut-infesting tephritid *Rhagoletis juglandis* that had been exposed to a fruit stimulus during early adulthood (Carsten and Papaj, 2005). Fruit experience could provide information to a foraging female on the quality and availability of host fruit in a patch (Carsten and Papaj, 2005). In our experiments, it is possible that females that were continuously exposed to fruit stimuli perceived that high quality oviposition resources were continually accessible and therefore mated promptly. Under such a scenario, females could assign more time to oviposition with associated fitness advantages. However, this argument does not explain why fruit-exposed females accepted more quickly and had shorter copulations with heterotypic males than with homotypic males. Further studies are needed to dissect the effect of natural stimuli on female choosiness.

In conclusion, males and females react to environmental cues with distinct sex-specific innate behaviours. While the space available during early development has more profound effects than other environmental conditions on the behaviour of male flies, the presence of host fruit and leaves influenced female behaviour. We suggest that there exists a minimal area for males to establish their territories and to reduce male–male aggressive interactions that negatively affects their sexual attractiveness. Differences in the response of both sexes to environmental enrichment offer an opportunity to investigate those factors that affect male sexual success and female choosiness. These characteristics are especially enticing for the study of lekking mating systems where tephritid flies have received great attention (Shelly and Whittier, 1997).

The results of the present study should serve to caution researchers working on laboratory kept animals, because the conditions under which animals are confined to before running a behavioural test, could greatly affect the outcomes of the experiments. Specifically, the results of studies on mating behaviour and sexual selection could be skewed or altered by the animal's early experience (Hebets, 2003; Siwicki et al., 2005; Svetec and Ferveur, 2005; Svetec et al., 2005a; Svetec et al., 2005b). This has particular relevance for the fruit fly quality-control methods of SIT programmes. Cages in which wild flies are held before the tests, should contain flies at low densities and also include natural stimuli to avoid the negative effects of crowding on their behavioural patterns and to favour female natural choosiness. A better perspective of the performance of mass-reared males could be obtained if they compete with wild males whose behaviour has not been influenced by laboratory conditions (Meza-Hernández and Díaz-Fleischer, 2006).

Our results also suggest that the negative effects of laboratory rearing may be mitigated by the careful design of housing conditions. Facilities should hold insects at low densities or redesign cages to maximise the surface area:volume ratio to avoid the negative effects of crowding on natural behavioural patterns. In the case of mass-reared flies, we argue that the quality and effectiveness of sterile flies released in SIT programmes could be dramatically increased by providing insects with adequate space. Moreover, packing systems in which adult insects emerge into crowded cages or bags, where they may stay for up to six days prior to release in nature, could overshadow many of the efforts to improve the earlier stages of the mass-rearing process.

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