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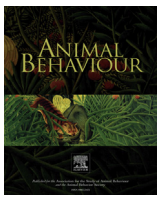

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Sex pheromones are not always attractive: changes induced by learning and illness in mice

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A male-specific major urinary protein named darcin is attractive to female mice, *Mus musculus*, stimulates a learned attraction to volatile components of a male's urinary odour and induces spatial learning. In this article we show that darcin also induces learned attraction for a previously neutral olfactory stimulus (the odorant isoamyl acetate), acquired by repeated presentation of both stimuli together. We hypothesize that this is a case of olfactory–vomeronasal associative learning, in which darcin acts as the unconditioned reinforcer. However, the presence of darcin is not always attractive to adult female mice. Urine from males parasitized by the nematode *Aspiculuris tetraptera* has no attractive value for females, despite apparently normal presence of darcin. The loss of attractive value may be due to unknown infection-derived chemicals whose detection overrides the attraction induced by darcin, or prevents detection of darcin by other (unknown) mechanisms. Other cases in which male urine (and thus the presence of darcin) does not induce attraction are discussed, namely in lactating females, which respond with aggression towards intruder males, and in prepubertal females, which show aversive responses towards unfamiliar male urine. Thus, although the darcin sex pheromone induces attraction in adult female mice that does not need to be learned, such innate pheromonal responses can be modulated by the physiological or health status of the sender and receiver. This provides a degree of flexibility in response to pheromonal signals, but such that individuals of the same class or status still share consistent predictable responses to improve their reproductive fitness. Further, by readily inducing the same response towards other odorants through associative learning, pheromones can also target responses flexibly towards odour signatures at an individual-specific level.

Pheromones were originally defined as 'substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behavior or a developmental process' (Karlson & Luscher, 1959, p. 55). Although this definition has been very useful for more than 50 years (Wyatt, 2009), the response to pheromones (at least in mammals) may vary depending on a number of factors, including the previous experience or the hormonal status of the receiver (Wyatt, 2010). In rodents, in which olfactory stimuli play a key role in many aspects of sociosexual behaviours (Brennan & Kendrick, 2006), experience with social chemical signals can influence later responses. For example, chemical signals present in urine of male mice, *Mus musculus*, detected through the vomeronasal system, have reinforcing properties able to induce appetitive associative learning, in such a way that other volatiles present in urine may become secondary attractive odorants (Martinez-Ricos, Agustin-Pavon, Lanuza, & Martinez-Garcia, 2007, 2008; Moncho-Bogani, Lanuza, Hernandez, Novejarque, & Martinez-Garcia, 2002; Ramm, Cheetham, & Hurst, 2008). Therefore, the behavioural response elicited by pheromones may also be seen as a learned response to stimuli previously associated with the pheromones, so that these stimuli acquire pheromone-like properties (Martinez-Garcia et al., 2009). Recently, a male-specific urinary protein named darcin has been shown to be able to induce this kind of olfactory learning (Roberts et al., 2010) and also spatial learning (Roberts, Davidson, McLean, Beynon, &

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Hurst, 2012). Since odours are easily associated with either positive or negative experiences (Herz & Cupchik, 1995), we hypothesized that darcin would be able to induce a secondary attraction by association with a neutral odorant (not present in urine), and to test this hypothesis we performed experiment 1, described below.

Notably, though, the behavioural response of females to the reinforcing properties of darcin (and maybe other male sexual pheromones) is affected by their endocrinological status. On the one hand, the preference of female mice for male-derived chemicals has been shown to appear with puberty, whereas prepubertal females display an aversive response to chemical signals from unfamiliar adult males (Drickamer, 1989; Mucignat-Caretta, Caretta, & Baldini, 1998). We do not yet know whether hormonal changes at puberty modify the behavioural response to darcin, changing it from aversion (shown by prepubertal females) to attraction (shown by postpubertal females), or whether other airborne components induce aversion to urine from unfamiliar adult males that prevents contact and delivery of this involatile protein pheromone to receptors. On the other hand, during lactation females show aggressive responses towards unfamiliar male intruders (and to a lesser extent also towards female intruders), to protect their pups (maternal aggression, Rosenson & Asheroff, 1975). Maternal aggression is low towards castrated males, and depends on the vomeronasal organ, as revealed by lesion (Bean & Wysocki, 1989) and gene knockout studies (Chamero et al., 2011). Hence, maternal aggression is elicited by the vomeronasal detection of testosterone-dependent chemical stimuli that may include darcin. Indeed, recent results from our laboratory indicate that the attractive pheromone darcin is also able to induce maternal aggression (Martin-Sanchez et al., 2014). Therefore, the female's hormonal status during lactation induces unknown changes in the neural circuitry processing darcin that alter the behavioural response to this pheromone from attraction to aggression.

In addition to the endocrinological changes at puberty and lactation that alter the behavioural response of females, previous studies have shown that viral infections or parasitosis in male mice used as donors of urine or bedding result in a lack of attractiveness of the urine (or soiled bedding) for females (e.g. Kavaliers, Choleris, & Pfaff, 2005; Penn et al., 1998). This lack of attractiveness may result from a loss of darcin expression as a result of the infection, or to the presence of unknown infection cues in the urine of infected males. To test these possibilities, we performed preference tests (experiment 2) using urine from males parasitized by the nematode *Aspiculuris tetraptera* versus (healthy) female urine, and tested whether the urine of parasitized males contains darcin.

EXPERIMENT 1: INDUCING ATTRACTION FOR A NEUTRAL ODORANT

Methods

Subjects

For the present study, 15 adult female mice (12–16 weeks) of the CD1 outbred strain were used (Janvier Labs, Le Genest-Saint-Isle, Saint-Berthevin Cedex, France). Treatment of these and the other animals used in experiments 1 and 2 complied with the European Union Council Directive of June 3 2010 (6106/1/10 REV1), according to which procedures were approved by the Committee of Ethics on Animal Experimentation of the University of Valencia (protocol number A1283764105250). Procedures also adhered to the ASAB/ABS Guidelines for the Use of Animals in Research.

The females were sexually naïve and had never been exposed to chemical signals from sexually mature males. To achieve this, pregnant females were housed in a clean room without males, in standard macrolon transparent cages with a wire lid (21.5 × 46.5 cm and 14.5 cm high, ref. 1000, Panlab, Barcelona, Spain) filled with soft wood bedding (Souralit S.L., ref. 3000, Barcelona, Spain), provided with nesting material (shredded paper) and enriched with cardboard tubes. The room was maintained at 22–24 °C, 60–80% relative humidity and a 12:12 h light:dark cycle, with lights on at 0800 hours. Food (Teklad Global 14% Protein Rodent Maintenance Diet, Harlan, ref: 2014) and water were available ad libitum. Nineteen days after delivery (well before puberty, which usually takes place about 6 weeks of age, see Silver, 1995), pups were sexed and males were removed. Female siblings were kept in a clean room in complete absence of adult male chemical signals, in groups of five or six per cage (the stock housing conditions in the experimental room were the same as described above for the pregnant females). Food and water were available ad libitum except during the 5 min preference tests and the 15 min training sessions. Welfare assessment took place during cage cleaning, and included noninvasive indicators. In the neonates, skin colour, activity and presence of the milk spot were observed; at weaning and in the adult, general appearance, size, coat condition, posture, gait, activity levels, interaction with the environment and clinical signs were observed (Wells et al., 2006). After weaning, animals were only manipulated for cage cleaning once a week. Since general appearance and size were evaluated as normal, no further care was necessary. Mice were handled following the standard practice of picking them up by gently holding the base of the tail and helping them onto the handler's arm, avoiding holding them in the air. All procedures involved in this study were noninvasive behavioural tests. At the end of the experiments, animals were euthanized with an intraperitoneal overdose of sodium pentobarbital (92 mg/kg), as indicated in the approved protocol (cited above). The male siblings were either used for anatomical studies (protocols approved by the Committee of Ethics on Animal Experimentation of the University of Valencia, under the same reference number A1283764105250; published elsewhere, Otero-Garcia et al., 2014) or euthanized as described above.

Stimuli

We chose two odorants that have frequently been used as neutral olfactory stimuli in the literature: isoamyl acetate (e.g. Angely & Coppola, 2010; Panreac, Barcelona, Spain) and citralva (e.g. Martinez-Ricos et al., 2007; geranonitrile, 3,7-dimethyl-2,6-octadiene-1-nitrile, International Flavours and Fragrances, Ventos, Barcelona, Spain). Both odorants were diluted 1:1000 in phosphate buffer (0.01 M) with 0.01% Triton X-100. In a pilot test of olfactory preference isoamyl acetate and citralva were investigated equally.

Preference tests

Animals were habituated to handling and to the test cage over 3 days, 10 min per day, between 1500 and 2000 hours. Preference tests were performed in cages measuring 25 × 50 cm and 30 cm high. Two 4 × 4 cm pieces of filter paper each impregnated with 5 µl of one of the stimulus odorants were presented on opposite sides of the cage. These impregnated papers were fixed to the bottom of the cage with a metallic cover, leaving exposed a circular area (diameter = 3.5 cm) that allowed direct nasal contact with the paper but prevented the animals gnawing or removing it.

For this olfactory preference test (citralva versus isoamyl acetate) females were released in the centre of the cage, the experimenter left the room, and the behaviour was video recorded for 5 min. The time that the animal spent in the circular area covered by the paper was measured by tracking animals automatically using the video analyser software Smart 2.5 (Panlab, Barcelona, Spain; see Fig. 1a). Since we observed that the animals lost interest in the olfactory stimuli at the end of the test, we restricted the analysis of the data to the first 4 min.

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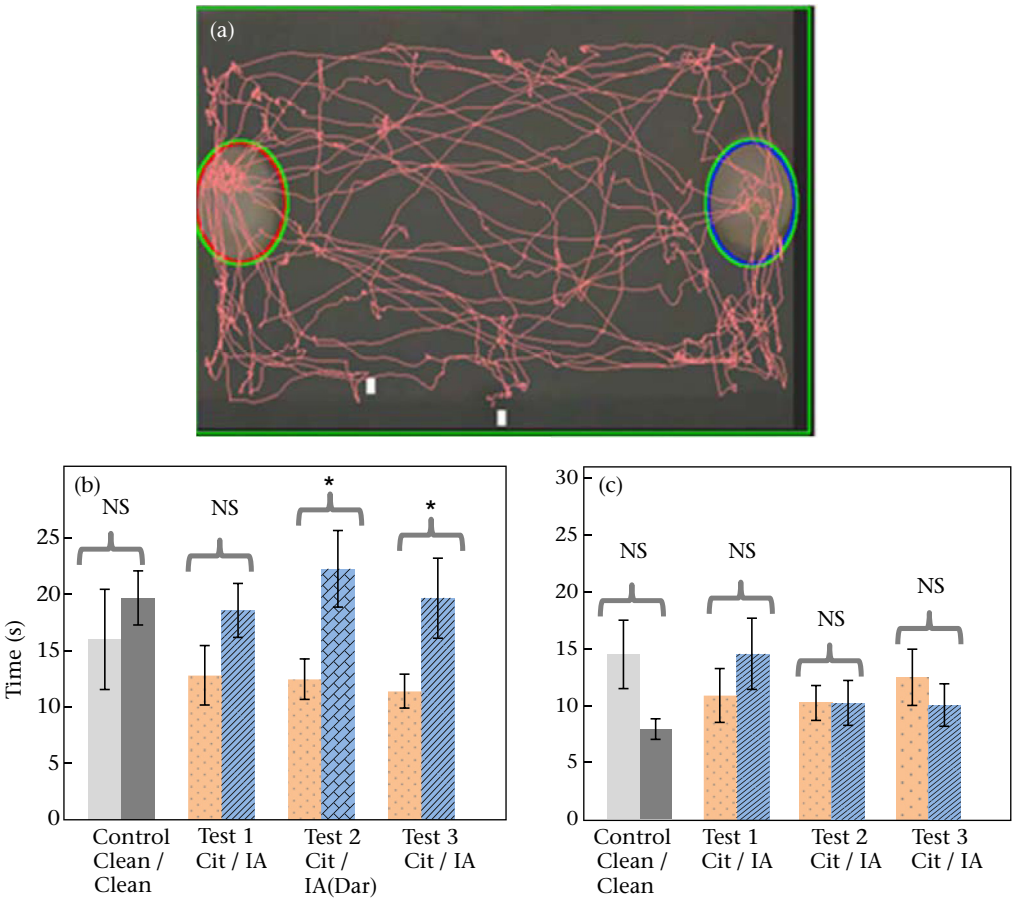

Figure 1. (a) Example video track of the exploratory behaviour of one animal in an odour preference test (citralva versus isoamyl acetate). The white pieces of scented paper are located inside the areas of measure. (b, c) Time (mean ± SE) spent by female mice in the areas where the odorant stimuli were presented, in the (b) experimental (darcin group) and (c) control groups. Control: clean pieces of paper (grey bars); cit: citralva area (stippled bars); IA: isoamyl acetate area (hatched bars). In test 2 in the experimental group, the isoamyl acetate-scented paper was also impregnated with r-darcin (IA(Dar); cross-hatched bar). Asterisks indicate significant *P* values in the analysis of the simple effects of the factor Side in each of the tests.

Following the pretraining preference test, the females were run in a second test in which the isoamyl acetate-scented paper was also impregnated with 8 μl of recombinant darcin (r-darcin, diluted 1.1 μg/μl, Roberts et al., 2010). Over the next 4 days, four training sessions (one per day) were performed in which a piece of paper (not fixed) scented with 5 μl of isoamyl acetate and 8 μl of r-darcin was presented to the females for 15 min/day in the centre of a different cage (29 × 15 cm and 29.8 cm high), and thus in a different context to that of the preference tests. Finally, a post-training olfactory preference test was performed (citralva versus isoamyl acetate) identical to the pretraining test described above. The location (left or right) of the citralva- and isoamyl acetate-scented papers was decided randomly at the beginning of the experiment and kept fixed for all the animals and tests.

After finishing this experiment, we wanted to eliminate the possibility that repeated presentations of the odorant, by themselves, induced preference for this familiar stimulus (against the other odorant, which was less familiar). To test this, we performed a second (control) experiment ($N = 12$, 16 weeks of age, Janvier Labs, Saint-Berthevin Cedex, France) identical to the previous one, except for the absence of darcin in the preference test and training sessions. Since females of this control experiment were not going to be exposed to male-derived chemosignals, they were acquired from Janvier as young adults, without preventing their prepubertal exposure to male odours. Housing and care conditions were the same as described above.

Statistical analysis

The time spent in the area occupied by the scented paper was analysed with a repeated measures ANOVA with the Test (control; first odour preference test; darcin preference test; second odour preference test) and Side (citralva versus isoamyl acetate) as intrasubject factors. The normality of the data was first confirmed with a Kolmogorov–Smirnov test with Lilliefor's correction. Analyses were performed with the SPSS 15.0 software package.

Results

The results of the repeated measures ANOVA for the time spent within each of the circular areas (Side) during the four tests (control; first odour preference test; darcin preference test; second odour preference test; Fig. 1b) revealed significant main effects of Side ($F_{1,14} = 7.87$, $P = 0.014$), and a nonsignificant main effect of Test or Side * Test interaction ($F < 1$, $P > 0.6$ in both cases). The analysis of the simple effects of the factor Side in each test showed that both areas were equally investigated in the control (clean versus clean: $F < 1$, $P > 0.4$) and the first odour preference test (citralva versus isoamyl acetate: $F_{1,14} = 1.18$, $P = 0.16$). By contrast, females spent significantly more time in the area occupied by the paper scented with isoamyl acetate plus darcin ($F_{1,14} = 5.07$, $P = 0.032$). Finally, in the second (post-training) olfactory preference test (citralva versus isoamyl acetate), females again spent more time in the area occupied by the isoamyl acetate-scented paper ($F_{1,14} = 5.77$, $P = 0.031$).

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In the control group, in which the same procedure was used but no darcin was present, the results of the repeated measures ANOVA for time spent within each of the circular areas (Side) during the four tests (Fig. 1b) showed no significant main effects of Side ($F_{1,11} < 1$, $P = 0.51$), Test ($F_{3,9} < 1$, $P = 0.71$) or their interaction ($F_{3,9} = 2.06$, $P = 0.17$). The analysis of the simple effects of the factor Side in each test showed that both areas were equally investigated in all cases (clean versus clean: $F_{1,11} = 3.23$, $P = 0.1$; first odour preference test: $F_{1,11} = 1.02$, $P = 0.33$; second odour preference test: $F_{1,11} < 1$, $P = 0.99$; third odour preference test: $F_{1,11} = 1.37$, $P = 0.26$).

Discussion

The results of experiment 1 show that a neutral odorant, such as isoamyl acetate, which is not significantly preferred by female mice, becomes a preferred olfactory stimulus when presented together with the sexual pheromone darcin. The repeated presentation of the odorant, as shown by the control experiment, did not alter the original lack of preference between the two olfactory stimuli used in the present tests.

Darcin is a male-specific nonvolatile urinary protein (MUP20, MW 18 893 Da) previously shown to induce in females a learned olfactory preference for the particular pattern of urinary volatiles displayed by an individual mouse (Roberts et al., 2010). In addition, darcin can also induce spatial learning (female mice remember the location where it was presented in a test cage, Roberts et al., 2012). Regarding this, we should keep in mind that spatial learning was also likely to take place in the present experiment, since we ran a 5 min preference test in which darcin was present following the first citralva versus isoamyl acetate test. However, some relevant differences between the present experiment and those reported by Roberts et al. (2012) suggest a weaker role of spatial learning in the present case. First, we used a test cage with no internal spatial cues, with both sides of the cage being equal. Second, our test was 5 min long (the training session in Roberts et al., 2012, was 10 min long). Third, we used 8.8 μg of r-darcin, whereas 50 μg were used in Roberts et al. (2012). Finally, in the present experiment the females were subsequently exposed to isoamyl acetate-scented papers impregnated with darcin daily for 15 min over the next 4 days, with these sessions taking place in a very different context, before testing their learned response to the odorants. This provided abundant possibilities for the formation of odour–pheromone associations, whereas the opportunities for spatial learning were comparatively few. In any case, the possible role of spatial learning cannot be discarded, and future experiments should confirm the induction of odour–pheromone learning suggested by the present results.

Previous work has shown that isoamyl acetate can be used as a conditioned stimulus in an aversive learning task, associating it with lithium chloride (Kay & Nyby, 1992), and therefore this olfactory stimulus can be conditioned to be either aversive or attractive. We hypothesize that in the present case of associative learning the conditioned stimulus (isoamyl acetate) is detected by the olfactory system and the unconditioned stimulus (darcin) is detected by the vomeronasal system. Recent findings, using electrophysiological recordings in single vomeronasal neurons, have suggested that darcin is indeed detected through the vomeronasal system (Kaur et al., 2014). Olfactory and vomeronasal information are known to converge in several nuclei within the corticomedial amygdala (Cadiz-Moretti, Martinez-Garcia, & Lanuza, 2013; Kang, Baum, & Cherry, 2009, 2011; Pro-Sistiaga et al., 2007), where learning may take place. In addition, further intramygdaloid projections would allow the participation of the nuclei of the associative (basolateral) amygdala, as suggested by functional data obtained with the immediate early gene *c-Fos* (Moncho-Bogani, Martinez-Garcia, Novejarque, & Lanuza, 2005). A different pheromone that has been shown to induce olfactory learning is the rabbit mammary pheromone 2-methylbut-2-enal (2MB2) (Coureaud et al., 2006), although in this case the 2MB2 is probably detected by the main olfactory system, as indicated by functional studies using the Fos protein as a neural activity marker (Charra, Datiche, Gigot, Schaal, & Coureaud, 2013).

The induction of a learned preference for airborne urinary stimuli should take place in natural conditions when female mice explore the urine marks that males use to advertise their territory (Hurst & Beynon, 2004). The urine of males is enriched in several volatile molecules, such as farnesenes, 2-sec-butyl-4,5-dihydrothiazole and 3,4-dehydro-*exo*-brevicomin, which have been shown to be also detected by the vomeronasal organ (Leinders-Zufall et al., 2000) and may possess pheromonal activity on their own (see, for a review, Dulac & Torello, 2003). For example, the mixture of alpha and beta farnesenes was shown to be attractive to sexually naïve female mice but only when used in very high concentrations, while having no effect when used at a concentration that was double that of normal dominant male urine (Jemiolo, Xie, & Novotny, 1991). By contrast, farnesenes were preferred even at low concentrations by sexually experienced animals (Jemiolo et al., 1991). Although the previous chemosensory experience of the animals in these earlier experiments is unknown, the effects of sexual experience clearly indicate a role for learning. Similar remarks can be made in other cases of putatively identified pheromonal stimuli, such as (methylthio)methanethiol (MTMT, an attractive semiochemical present only in the urine of male mice, Lin, Zhang, Block, & Katz, 2005) and androstenone, a pheromone that facilitates expression of both attraction to the male and a receptive mating stance in oestrous female pigs (Dorries, Adkins-Regan, & Halpern, 1997). In both studies the female subjects had previous sexual experience (in the case of the female pigs most of them were multiparous). In the light of the results presented here, the pheromonal role of these semiochemicals should be re-evaluated at least using sexually naïve (if not chemosensory naïve) animals to understand the requirement for learning. In the same vein, the human steroid androstenone (5α-androst-16-en-3-one) has been proposed to function as a human sex chemosignal (see, for a review, Havlicek, Murray, Saxton, & Roberts, 2010). However, the hedonic value of androstenone was recently evaluated as a function of sexual experience (Knaapila et al., 2012), the odour being rated as unpleasant by women who reported never having experienced sexual intercourse, and as less unpleasant by those who reported being sexually experienced. Since humans do not have a functional vomeronasal organ (Meredith, 2001), in this case learning is likely to be mediated by the association of the olfactory cue with other kinds of rewarding stimuli related to sexual activity.

The phenomenon of pheromone-induced olfactory learning raises the question of whether a substance that gains its role as chemical signal by a learned association should be considered a pheromone, since it does not elicit a fixed (stereotyped) response (as required by the original definition of Karlson & Luscher, 1959) before learning takes place. However, in the case of the male-specific airborne urinary substances this olfactory–vomeronasal association would necessarily occur every time the female interacts with males or after encountering their urine marks. Moreover, females will form different olfactory–vomeronasal associations with each male, so that the learned response would be specific to that particular signature (Ramm et al., 2008). Under natural conditions, females are probably able to detect male-specific cues at a distance using the main olfactory system. Once the female locates the male (or his urine marks) the input through the vomeronasal organ (requiring direct contact with the source) would allow further information about this particular individual to be processed. Several

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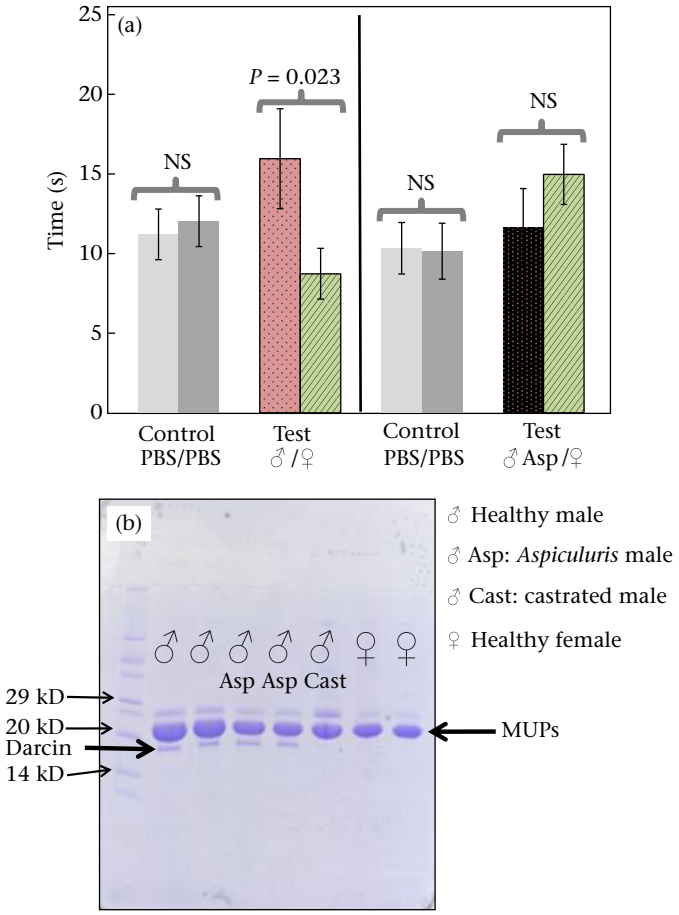

Figure 2. (a) Time (mean ± SE) spent by female mice in areas where urine stimuli were presented. PBS/PBS: control (grey bars); ♂/♀: urine of healthy males (stippled bar) versus females (hatched bar); ♂ Asp/♀: urine of parasitized males (dark stippled bar) versus females (hatched bar). The *P* value corresponds to pairwise comparison with Bonferroni correction. (b) SDS–PAGE of urinary protein for healthy male mice ($N = 2$), male mice parasitized with *Aspiculuris tetraptera* ($N = 2$), a castrated male mouse ($N = 1$) and females ($N = 2$). The 20 kDa band present in all cases corresponds to the molecular weight to the major urinary proteins. The small band with higher mobility (around 17 kDa), present in healthy and infected males, corresponds to the expected position of darcin.

of the male-specific urinary volatiles are also detected by the vomeronasal organ (Leinders-Zufall et al., 2000), but it is currently unknown why the detection of volatile signals by the vomeronasal organ requires direct contact with the stimulus, as indicated by both behavioural and electrophysiological evidence (Luo, Fee, & Katz, 2003; Moncho-Bogani et al., 2002; Ramm et al., 2008).

EXPERIMENT 2: DARCIN IN URINE FROM PARASITIZED MALES

Methods

Subjects and stimuli

For this experiment, 32 adult female mice (12–16 weeks) of the CD1 strain were used (Janvier Labs, Le Genest-Saint-Isle, Saint-Berthevin Cedex, France). As for experiment 1, females were sexually naïve and had never been exposed to chemical signals from sexually mature males. Females were housed in groups of five or six animals, with the same housing conditions (cages, bedding and food), manipulation and welfare assessment as those reported in experiment 1.

Urine from healthy adult CD-1 male and female mice was purchased from Janvier and kept frozen in aliquots until used. Urine from a small colony ($N = 4$) of male mice (also purchased from Janvier) naturally infected with the intestinal nematode *A. tetraptera* was collected as described by Kurien, Everds, and Scofield (2004). Briefly, animals were gently held by the scruff of the neck over a petri dish, from where urine was pipetted. Since the infection occurred naturally, the animals probably experience this level of parasitism in the wild. The presence of these parasites in male mice was detected with the routine sentinel vigilance system. The infected mice were euthanized (as described in the Methods for experiment 1) except for four animals that were kept for 5 days. During this time we collected their urine once a day. At the end of this 5-day period the four infected animals were also euthanized. The infected mice showed no external signs of infection, and general appearance, size, coat condition, posture, gait, activity levels, interaction with the environment and clinical signs appeared normal. Infected male mice were housed in pairs in standard macrolon cages with a wire lid (22.5 × 22.5 cm and 14.5 cm high, ref. 500, Panlab, Barcelona, Spain). The rest of the housing conditions were the same as described in experiment 1. To ensure the homogeneity of the stimulus across behavioural tests, urine from different males was mixed and stored in frozen aliquots of 50 µl. Infection with the parasite was assessed through the presence of eggs in faecal pellets and confirmed post mortem by checking for adult worms in the colon.

Preference tests

The test cage and habituation procedure were as described in experiment 1. Preference tests were performed in which female mice had to choose between two urine stimuli located in opposite sides of the cage. To do so, 10 µl of the stimulus urine was pipetted on one tip of a rectangular piece of filter paper (2 × 6 cm) that was attached to the wall so that the urine spots were 8 cm above the floor. Females were able to make direct nasal contact with the stimuli by standing on their hind legs. Following a control test, with PBS 0.1 M on both sides of the cage, the olfactory preference test (male versus female urine) was performed and recorded for 5 min as described for experiment 1. In one group of animals (randomly assigned, $N = 16$), urine of healthy males was presented on one side and female urine on the other. In the second group of females (randomly assigned, $N = 16$), urine of infected males was presented against female urine.

Statistical analysis

The time spent in a semicircular area (of a radius of 3.85 cm) around the filter paper was analysed with a three-way repeated measures ANOVA with Test (control, preference test) and Stimulus (male versus female urine) as intrasubject factors and Group (urine from healthy or infected males) as an intersubject factor. Significant interactions were further analysed by multiple pairwise comparisons with Bonferroni corrections. The normality of the data was first confirmed with a Kolmogorov–Smirnov test with Lilliefor's correction. Analyses were performed with the SPSS 15.0 software package.

Electrophoresis of urinary proteins

The pattern of bands corresponding to urinary proteins, separated according to their mass, was visualized using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Urine was diluted 1:1 with 2× denaturalization buffer (20 mM Tris pH 8.0, 5% SDS, 10% mercaptoethanol, 2 mM EDTA and 0.05% bromophenol blue) in a capped Eppendorf tube, vortexed to mix, boiled for 5 min and then centrifuged for 5 min at 10 000 rpm. The samples were then allowed to cool before sample loading. Using 1×

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denaturalization buffer, samples of male urine were brought to a final 1:6 dilution, whereas female urine samples were not further diluted (final dilution 1:2). This allowed direct comparison of male and female urine protein species, in spite of the difference in total urinary protein content between the sexes. Using a PhastGel system (General Electrics), electrophoresis was run under reducing conditions at a constant 200 V on a 20% polyacrylamide gel (PhastGel Homogeneous − 20, GE). Low range molecular weight markers (Sigmamarker low range, M3913, St Louis, MO, U.S.A.) were used for comparison. Following electrophoresis, protein bands were visualized using PhastGel Blue (0.1%) solution and differentiated in a solution of methanol:acetic acid:distilled H_2O (30:10:60 v/v/v).

Results

The results of the repeated measures ANOVA of time spent within the areas surrounding the stimulus during the tests (Fig. 2a) showed nonsignificant main effects of factors Test ($F_{1,30} = 1.9$, $P = 0.17$), Stimulus ($F_{1,30} < 1$, $P = 0.6$) or Group ($F_{1,30} < 1$, $P = 0.9$), and nonsignificant interactions between each pair of these factors (Stimulus∗Test: $F_{1,30} = 1.14$, $P = 0.29$; Test∗Group: $F_{1,30} = 2.43$, $P = 0.12$; Stimulus∗Group: $F_{1,30} < 1$, $P = 0.4$). However, there was a significant triple interaction (Stimulus∗Test∗Group: $F_{1,30} = 7.45$, $P = 0.01$). Further analysis of this triple interaction (Fig. 2a) with multiple pairwise comparisons with Bonferroni corrections showed that in both groups the two stimulus areas were investigated equally in the control condition, when saline buffer was present on both sides of the cage (healthy male group: $P > 0.7$; infected male group: $P > 0.9$). By contrast, females presented with urine of healthy males showed a clear preference for this stimulus over female urine ($P = 0.023$); females presented with urine of infected males spent more time next to the female urine, although the time spent next to each stimulus was not significantly different ($P = 0.27$).

To check for the presence of the sexual pheromone darcin in the urine of male mice infected with *A. tetraptera*, we performed an SDS–PAGE electrophoresis to compare proteins in healthy male urine, urine of infected males, urine of castrated (uninfected) males and female urine (Fig. 2b). The results showed that the pattern of protein bands in the urine of infected males was similar to that observed in the urine of healthy males, with a clearly visible band of higher mobility than other major urinary proteins that corresponds to darcin (Armstrong, Robertson, Cheetham, Hurst, & Beynon, 2005). By contrast, this band was not present in the urine from castrated males or in female urine, confirming previous results (Armstrong et al., 2005; Cheetham, Smith, Armstrong, Beynon, & Hurst, 2009).

Discussion

The learning process induced by darcin would allow females to discriminate between different males (and their urine marks) and consequently choose the one expressing more attractive features and refuse those less attractive or even with aversive features. In this regard, it has been shown that female mice are able to discriminate between infected and uninfected males and show a preference towards healthy males (see Kavaliers et al., 2005). The results of the present experiment suggest that females not only choose uninfected males against infected males, but also that the preference for male urine over female urine is lost if urine comes from parasitized males. This result is consistent with a previous report that showed that females show no attraction to the urine of males infected with influenza viruses when given a choice between infected urine and water (Penn et al., 1998). Since it is known that the attractive properties of male urine depend on the male-specific urinary protein darcin (Roberts et al., 2010), we tested whether the expression of this protein may have been lost in males infected with *A. tetraptera*. Although their urine was not attractive to females, darcin was present in the urine of these parasitized males apparently at normal levels, although further analyses would be required to confirm whether there are subtle quantitative differences. Although we cannot discount a small reduction in the expression of this protein, this is unlikely to explain the total lack of preference for male urine versus female urine that did not contain darcin (at least at a level that could be detected by electrophoresis). Changes in the amino acid sequence or conformation of the protein are very unlikely. Therefore, we can hypothesize that an infection cue exists in the urine of the parasitized animals, and that detection of this is able to override the attractive value of darcin. An alternative hypothesis, though, is that the cue of infection is volatile and can be detected at a distance; this detection may inhibit the vomeronasal pumping necessary to deliver high molecular weight molecules, such as darcin, to the vomeronasal organ (Meredith, Marques, O'Connell, & Stern, 1980; Wysocki, Wellington, & Beauchamp, 1980). In this case, darcin would simply not be detected by females.

Whether volatile or involatile (or both), the identity of the putative cue(s) of infection is unknown, as is its nature as an olfactory or vomeronasal stimulus. It has recently been demonstrated that the vomeronasal organ of mice expresses a different type of chemosensory receptor named FPR (formyl peptide receptors, Liberles et al., 2009; Riviere, Challet, Fluegge, Spehr, & Rodriguez, 2009). This type of receptor detects ligands related to the immune system (such the antimicrobial peptide CRAMP, lipoxin A4 or uPAR, see Riviere et al., 2009) in addition to formylated peptides produced by bacteria, therefore providing candidate receptors for the sensing of urinary infection cues; however, experimental evidence for this possibility is currently lacking.

GENERAL CONCLUSIONS

Although the attractive response that adult female mice show towards the male-specific pheromone darcin is innate, here we have shown that, on the one hand, the same behavioural response can be induced by nonpheromonal stimuli that have been associated with darcin through learning and, on the other, the presence of darcin does not induce an attractive response when present in the urine of male mice infected by an intestinal parasite.

The use of a species-specific pheromonal signal such as darcin in the context of mate attraction provides the considerable advantage of ensuring that adult females are reliably and sensitively attracted to the scent of males of their own species, regardless of any need for prior exposure to adult males or the opportunity to learn appropriate male-specific cues and responses during development. However, a signal that induced a fixed, inflexible response (regardless of the physiological state of the female or of the suitability as potential mates of males that express the signal) would reduce the ability of females to behave adaptively according to their current state or context, or to select males based on any criterion other than their ability to produce the pheromone signal. Instead, innate attraction induced by a simple pheromone signal in male urine is modulated in prepubertal or lactating females, and by additional information in male urine indicating their infection status. This provides a degree of flexibility in response, although individuals of the same status still share the same consistent response to the same signal relevant to their current reproductive state. However, we have also discovered that pheromones can be potent stimuli for associative learning, which means that a pheromone-induced response can be transferred flexibly to other associated stimuli according to the individual female's own

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experience, providing much greater flexibility in behaviour at an individual-specific level. Our findings here indicate that this learning is not constrained to odorants produced specifically by adult male mice but females will learn an attraction even to neutral odorants associated with the pheromone. Thus, pheromone-induced learning targets attraction to the associated odour signatures of individual males when they encounter darcin in their scent, but these learned signatures could include a broad range of odorants perhaps including those derived from the local environment and diet. Finally, this induction of learned attraction by a simple pheromone provides an interesting and tractable model to study learning and memory mechanisms further in an ethologically relevant context, and to understand the neural circuits that underlie sexual attraction.

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