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TITLE: Sex pheromones are not always attractive: changes induced by learning, puberty, lactation and illness in mice

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ABSTRACT

A male-specific major urinary protein named darcin is attractive to female mice, stimulates a learned attraction to volatile components of a male's urinary odour (Roberts et al., 2010, BMC Biol 8:75) and induces spatial learning (Roberts et al., 2012, Science 338:1462-5). In the present work we show that darcin also induces a learned attraction for a previously neutral olfactory stimulus (the odorant isoamyl acetate). However, the attractive properties of darcin may change as a function of female physiological state. For example, during the period of lactation female mice display aggressive behaviour against intruders, which is enhanced when confronted with adult males. Therefore, the endocrinological status of the females radically changes the behavioural response. The situation at puberty is somewhat similar: prepubertal females avoid adult male chemical signals, whereas post-pubertal females show attraction for these same signals. In addition, we report another situation in which the presence of darcin is not attractive to adult female mice. Urine of males parasitized by the intestinal nematode *Aspiculuris tetraptera* shows no attractive value for female mice, despite apparently normal presence of darcin. In this case, the loss of the attractive value is not due to physiological changes in the receptor females but is likely to be due to the presence of unknown signals of infection whose detection overrides the attraction normally induced by darcin.

KEY WORDS: vomeronasal, olfactory, sexual attraction, learning, maternal aggression, puberty, illness cues.

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). 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). Moreover, the behavioural response elicited by pheromones may also be observed as learned responses to stimuli previously associated with the pheromones, so that these stimuli acquire pheromone-like properties (Moncho-Bogani, Lanuza, Hernández, Novejarque, & Martínez-García, 2002). Here, we review relevant examples in mice, in which the attractive value of male sexual pheromones is transferred to a neutral stimulus or changes either as a function of the hormonal condition of the receiver females or the health status of scent donor males. In addition, we include data from two experiments giving support to the ideas presented in this review.

Pheromone-induced olfactory learning

Odours are easily associated with either positive or negative experiences, becoming secondary attractive or aversive stimuli that strongly influence behavioural responses in many mammalian species, including humans (Herz & Cupchik, 2005). In rodents, olfactory stimuli play a key role in many aspects of socio-sexual behaviours (Brennan & Kendrick, 2006). The experience with social chemical signals influences later responses. For example, chemical signals present in urine of male mice, detected through the vomeronasal system, have reinforcing properties able to induce appetitive associative learning (Martínez-Ricós, Agustín-Pavón, Lanuza, & Martínez-García,

2007, 2008; Moncho-Bogani et al., 2002; Ramm, Cheetham, & Hurst, 2008), in such a way that other volatiles present in urine may become secondary attractive odorants (Martínez-García et al., 2009). More 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, & Hurst, 2012). We hypothesized that darcin would be able to induce a secondary attraction by association with a neutral odorant (not present in urine). To test this possibility, we performed the following experiment.

EXPERIMENT 1: Inducing attraction for a neutral odorant by association with darcin

Material and 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 the animals employed in the experiments reported in this paper complied with the European Union Council Directive of June 3rd, 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 x 46.5 x 14.5 cm, 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% RH 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 (early before puberty), pups were sexed and males were removed. Their female siblings were brought to a clean room in complete absence of adult male chemical signals, where they were kept in groups of 5-6 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 preference tests (five-minute long) and the training sessions (15-minute long). Welfare assessment took place during cage cleaning, and included non-invasive 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 handle 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 to hold them in the air. All procedures involved in this study were non-invasive 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-García et al., 2014) or euthanized as described above.

Stimuli

We chose two odorants that have been used frequently as olfactory stimuli in the literature: isoamyl acetate (e.g., Angely & Coppola, 2010; Panreac, Barcelona, Spain) and citralva (e.g., Martínez-Ricós et al., 2007; geranonitrile, 3,7-dimethyl-2,6-octadiene-1-nitrile, kindly provided by 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 we proved that 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 15:00 and 20:00 hours. Preference tests were performed in 25 x 50 x 30 cm cages. A 4 x 4 cm piece of filter paper impregnated with 5 µl of one of the stimulus odorants was presented in each opposing side 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 to gnaw or remove it.

For this olfactory preference test (citralva vs isoamyl acetate) females were released in the centre of the cage, the experimenter left the room, and the behaviour was videotaped 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 four minutes.

Following the pre-training 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 four 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 during 15 min/day in the centre of a different cage (29 x 15 x 29.8 cm), and thus in a different context to that of the preference tests. Finally, a post-training olfactory preference test was performed (citralva vs isoamyl acetate) identical to the pre-training 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 discard the possibility that repeated presentations of the odorant, by themselves, induced preference for this familiar stimulus (against the other odorant, which is less familiar). To test this idea, 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, 1st odour preference test; darcin preference test; 2nd odour preference test) and SIDE (citralva vs. isoamyl acetate) as intra-subjects factors. The normality of the data was previously confirmed with a Kolmogorov-Smirnov test with Lillieford'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; 1st odour preference test; darcin preference test; 2nd odour preference test; Figure 1B) revealed significant main effects of SIDE ($F_{1,14} = 7.87$, $p = 0.014$), and non-significant main effect of TEST or the SIDE x 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 1st odour preference test (citralva vs. isoamyl acetate, $F_{1,14} = 1.18$, $p = 0.16$). By contrast, the 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 2nd (post-training) olfactory preference test (citralva vs. isoamyl acetate), females again spent more time in the area occupied by the isoamyl acetate-scented paper ($F_{1,14} = 5.77$, $p = 0.031$).

In the control group, in which the same procedure was used but no darcin was present, the results of the repeated measures ANOVA for the time spent within each of the circular areas (SIDE) during the four TESTS (Figure 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 the present experiments show that a neutral odorant, such as isoamyl acetate, which is not significantly preferred by female mice, becomes a preferred olfactory stimulus by presenting it 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 non-volatile urinary protein (MUP20, MW 18893 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 also 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 is also likely to take place in the present experiments, since we ran a five-minute preference test in which darcin was present following the first citralva vs. isoamyl acetate test. However, some relevant differences between the present experiments and those reported by Roberts et al. (2012) suggest a weaker role of spatial learning in the present case. Firstly, we used a test cage with no internal spatial cues, with both sides of the cage being equal. Secondly, our test was five minute long (the training session in Roberts et al., 2012, was 10 minutes long). Thirdly, we used 8 µl of r-darcin, while 50 µl were used in Roberts et al. (2012), at approximately the same concentration. And finally, in the present experiment the females were later exposed to isoamyl acetate scented papers impregnated with darcin daily for 15 min over the next four days, with these sessions taking place in a very different context. This provided abundant possibilities for the formation of odour-pheromone associations, whereas the opportunities for spatial learning were comparatively more reduced. 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 (since the animals need direct contact with the stimulus to show either innate attraction or learned associations, Roberts et al., 2010). Olfactory and vomeronasal information are known to converge in several nuclei within the corticomedial amygdala (Cádiz-Moretti, Martínez-García, & 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, Martínez-García, 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 likely detected by the main olfactory system, as indicated by functional studies using the Fos protein as 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 maybe 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 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 estrous 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 reevaluated 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, 1953) 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 of male urine marks. Moreover, females will encounter 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 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).

Turning attraction into aggression: male-specific vomeronasal cues elicit maternal aggression

As stated above, the attraction that females display for male urine is innate. However, during lactation females show aggressive responses towards male (to a lesser extent also towards female) intruders, to protect their pups (maternal aggression, Rosenson & Asheroff, 1975). Maternal aggression towards intruders is observed in the first two weeks after delivery, and disappears gradually onwards (Lonstein & Gammie, 2002). Maternal aggression is low towards castrated males, and vomeronasal organ removal in females prior to mating or after parturition eliminates later maternal aggression in mice

(Bean & Wysocki, 1989). Therefore, for the female to show maternal aggression the vomeronasal detection of testosterone-dependent chemical stimuli from males is required. This raises the question of whether some of the major urinary proteins, whose synthesis is testosterone-dependent and have been shown to be the vomeronasal stimuli mediating aggression between males (Chamero et al., 2007), are also the vomeronasal stimuli that elicit maternal aggression in lactating females. Preliminary data in our laboratory suggest that this is indeed the case (Martín-Sánchez et al., 2013), and that the attractive pheromone darcin is also able to induce maternal aggression. Therefore, the hormonal status of the lactating females induces changes in the neural structures processing darcin (probably not in the vomeronasal organ, although direct evidence of this is lacking). These changes may take place in the amygdalo-hypothalamic circuits involved in the aggressive response (Nelson & Trainor, 2007), but experimental evidence of this hypothesis is needed. Notably, lactating females are not aggressive towards familiar mates (Lonstein & Gammie, 2002), and therefore the response also depends on the learned identity of the individual male. Individual recognition in mice is mediated by the pattern of major urinary proteins (Hurst et al., 2001), and therefore the detection of the pattern of major urinary proteins corresponding to the familiar male should inhibit the aggressive response. Social recognition involves changes of gene expression (in particular oxytocin, vasopressin and steroid hormone receptors) in the amygdala and the hypothalamus (Clipperton-Allen et al., 2012), giving support to the hypothesis of the control of the aggressive responses by amygdalo-hypothalamic circuits (Bosch & Neumann, 2012).

Turning aversion into attraction: changes at puberty inducing attraction to male pheromones

Although the sexual attraction that female mice display for the male sexual pheromone darcin does not require learning (Roberts et al, 2010), it seems to appear with puberty, since pre-pubertal females display an aversive response to chemical signals from unfamiliar adult males (Drickamer, 1989; Mucignat-Caretta, Caretta, & Baldini, 1998). The biological bases of this change are unknown, although clearly the gonadal steroids underlying the pubertal changes should play a relevant role. In fact, the vomeronasal organ, likely involved in the detection of protein sex pheromones (such as darcin, Roberts et al., 2010; and exocrine gland-secreting peptide 1, Haga et al., 2010), as well as most of the neural centres of the vomeronasal system are sexually dimorphic (Segovia & Guillamon, 1993). This suggests a role for sexual steroids in development and differentiation of the vomeronasal system. Moreover, gonadal steroids also regulate the expression of some vomeronasal receptors (Alekseyenko, Baum, & Cherry, 2006), raising the possibility that sex steroids induce the expression of particular (currently unknown) receptors for male pheromones. In fact, there is some evidence of estradiol effects on the induction of c-Fos in the vomeronasal organ of female mice by male-soiled bedding (Halem, Cherry, & Baum, 1999). In addition, the presence of estradiol receptors is very important in the secondary vomeronasal centres, namely the medial amygdala, the posteromedial cortical amygdala and the posteromedial bed nucleus of the stria terminalis (Mitra et al., 2003). Therefore, the pubertal changes underlying the induction of the attraction of female mice for male pheromones may also take place in the amygdaloid circuits processing vomeronasal information.

From attraction to aversion: illness-derived chemicals and avoidance of conspecifics

During the last years, our research has been focused on the attractive properties of sexual pheromones and neurobiological foundation of this phenomenon. Occasionally we observed that viral infections or parasitosis in male mice used as donors of urine or bedding resulted in a lack of attractiveness of the urine (or soiled bedding) for females. This fits previous studies reporting avoidance of males by females when males were infected with viruses or parasites (e.g. Penn et al. 1998; Kavaliers et al., 2005). To check the response of female mice to the urine of infected males experimentally, we performed preference tests (Experiment 2) using urine from males parasitized by the nematode *Aspiculuris tetraptera* against (healthy) female urine. In addition, since darcin appears to be the both necessary and sufficient to make male urine attractive to females, we tested whether the urine of parasitized males contains darcin.

EXPERIMENT 2. Behavioural response of female mice to the urine of infected males

Material and Methods

Subjects and stimuli

For this experiment, 32 adult female mice (12-16 weeks) of CD1 strain were used (Janvier, 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 5-6 animals, with the housing conditions (cages, bedding and food), manipulation and welfare assessment being the same 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 *Aspiculuris tetraptera* was collected as described by Kurien, Everds & 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 can 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 sacrificed 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 sacrificed. 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 x 22.5 x 14.5 cm, ref. 500, Panlab, Barcelona, Spain). The rest of 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 the 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 were pipetted on one of the tips of a rectangular piece of filter paper (2 x 6 cm) that was attached to the wall so that the urine spots were 8 cm above the floor. The females were able to have direct nasal contact with the stimuli by standing on the hind legs. Following a control test, with PBS 0.1M on both sides of the cage, the olfactory preference test (male vs female urine) was performed and recorded for five minutes 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 side. 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 two-way repeated-measures ANOVA with TEST (control, preference test) and STIMULUS (male vs. female urine) as intra-subject factors, and GROUP (urine from healthy or infected males) as an inter-subject factor. Significant interactions were further analysed by multiple pair-wise comparisons with Bonferroni corrections. The normality of the data was previously confirmed with a Kolmogorov-Smirnov test with Lillieford'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 visualised using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Urine was diluted 1:1 with 2x denaturalization buffer (20mM Tris pH 8.0, 5% SDS, 10% mercaptoethanol, 2mM 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 10000 rpm. The samples were then allowed to cool before sample loading. Using 1x denaturalisation 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 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, USA) were used for

comparison. Following electrophoresis, protein bands were visualised using Phast Gel 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 (Figure 2A) showed non-significant 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 non-significant interactions between each pair of these factors (STIMULUS x TEST: $F_{1,30} = 1.14$, $p = 0.29$; TEST x GROUP: $F_{1,30} = 2.43$, $p = 0.12$; STIMULUS x GROUP: $F_{1,30} < 1$, $p = 0.4$). However, there was a significant triple interaction (STIMULUS x TEST x GROUP: $F_{1,30} = 7.45$, $p = 0.01$). Pos-hoc analysis of this triple interaction (Figure 2A) 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 the nematode *Aspiculuris tetraptera*, we performed a SDS-PAGE electrophoresis to compare proteins in healthy male urine, urine of infected males, urine of castrated (non-infected) males and female urine (Figure 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 et al., 2005). By contrast,

this band was not present in the urine from castrated males or in female urine, confirming previous results (Armstrong, Robertson, Cheetham, Hurst, & Beynon, 2005; Cheetham, Smith, Armstrong, Beynon, & Hurst, 2009).

Discussion

It has been shown previously that female mice are able to discriminate between infected and non-infected males and show a preference towards healthy males (see Kavaliers, Choleris, & Pfaff, 2005). The results of the present experiment suggest that females not only choose non-infected males against infected males, but also that the preference for male urine over female urine is lost if urine comes from parasitized males. Since it is known that the attractive properties of male urine depend on the presence of a male-specific urinary protein named darcin (Roberts et al., 2010), which acts as a sexual pheromone, we tested whether the expression of this protein may have been lost in the infected males. The results show that darcin is present in the urine of parasitized males apparently at normal levels, though further analyses would be required to confirm whether there are quantitative differences. Although we cannot discard 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-acidic 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 darcin, to the vomeronasal organ (Meredith, Marques, O'Connell, & Stern, 1980;

462 Wysocki, Wellington, & Beauchamp, 1980). In this case, darcin would simply not be
463 detected by females.
464 Whether volatile or involatile (or both), the identity of the putative cue(s) of infection is
465 unknown, as it is its nature as olfactory of vomeronasal stimulus. It has recently been
466 demonstrated that the vomeronasal organ of mice expresses a different type of
467 chemosensory receptor named FPR (formyl peptide receptors, Liberles et al., 2009;
468 Riviere, Challet, Fluegge, Spehr, & Rodriguez, 2009). This type of receptor, in addition
469 to formylated peptides produced by bacteria, detects ligands related with the immune
470 system (such the antimicrobial peptide CRAMP, lipoxin A4, or uPAR, see Riviere et
471 al., 2009), and therefore are good candidates for sensing of urinary infection cues,
472 although experimental evidence for this possibility is currently lacking.
473
474 GENERAL CONCLUSSIONS
475 Although the attractive response that adult female mice show towards the male-specific
476 pheromone darcin is innate, here we review four examples in which either the
477 behavioural response can be induced by non-pheromonal stimuli or the presence of the
478 pheromone does not induce the normal attractive response, due a non-receptive
479 hormonal status (pre-pubertal or lactating females) or to the putative presence of
480 infection cues in the male urine.
481 The induction of learned attraction by a pheromone is an interesting model to study
482 learning and memory mechanisms in an ethologically relevant context. In addition,
483 changes in response to darcin induced at puberty and during lactation provide
484 unexplored models of neural plasticity with clear and robust behavioural correlates,
485 which may be helpful to understand the neural circuits that induce both attractive and
486 aggressive responses.

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677 FIGURE LEGENDS

678 Figure 1. **A**: Example videotrack of the exploratory behaviour of one animal in an
679 odour preference test (citralva versus isoamyl acetate). The white pieces of scented
680 paper are visible inside the areas of measure. **B**: Time (mean ± SE) spent by female
681 mice in the areas where the odorant stimuli were presented, in the experimental (left
682 panel, darcin group) and control (right panel) groups. Grey bars in the control test
683 represent clean pieces of paper. Orange bars represent time spent in the citralva area.
684 Green bars represent time spent in the isoamyl acetate area. In test 2 in the experimental
685 group, the isoamyl acetate-scented paper was also impregnated with r-darcin. Females
686 showed a significant innate preference to remain near to the r-darcin sample ($p = 0.032$)
687 and a learned preference to stay next to the isoamyl acetate-scented paper in the post
688 training preference test ($p = 0.031$). In the absence of darcin, no preference appeared for
689 any odorant.

690

691 Figure 2. **A**: Time (mean ± SE) spent by female mice in areas where urine stimuli
692 were presented. The left side corresponds to females that were presented with urine of
693 healthy males versus females. Grey bars represent time spent near pieces of paper with a
694 sample of PBS (control). The green bar represents time spent near urine of healthy
695 males, and purple bar the time spent near female urine. The right side corresponds to
696 females that were presented with urine of parasitized males versus females. Grey bars
697 represent the control test. Females showed no preference for the urine of infected males
698 (dark red bar) versus female urine (purple bar). **B**: SDS-PAGE of urinary protein for
699 healthy male mice ($n = 2$), male mice parasitized with *Aspiculuris tetraptera* ($n = 2$),
700 castrated male mice ($n = 1$) and females ($n = 2$). The 20 kDa band present in all cases
701 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.

Figure 1

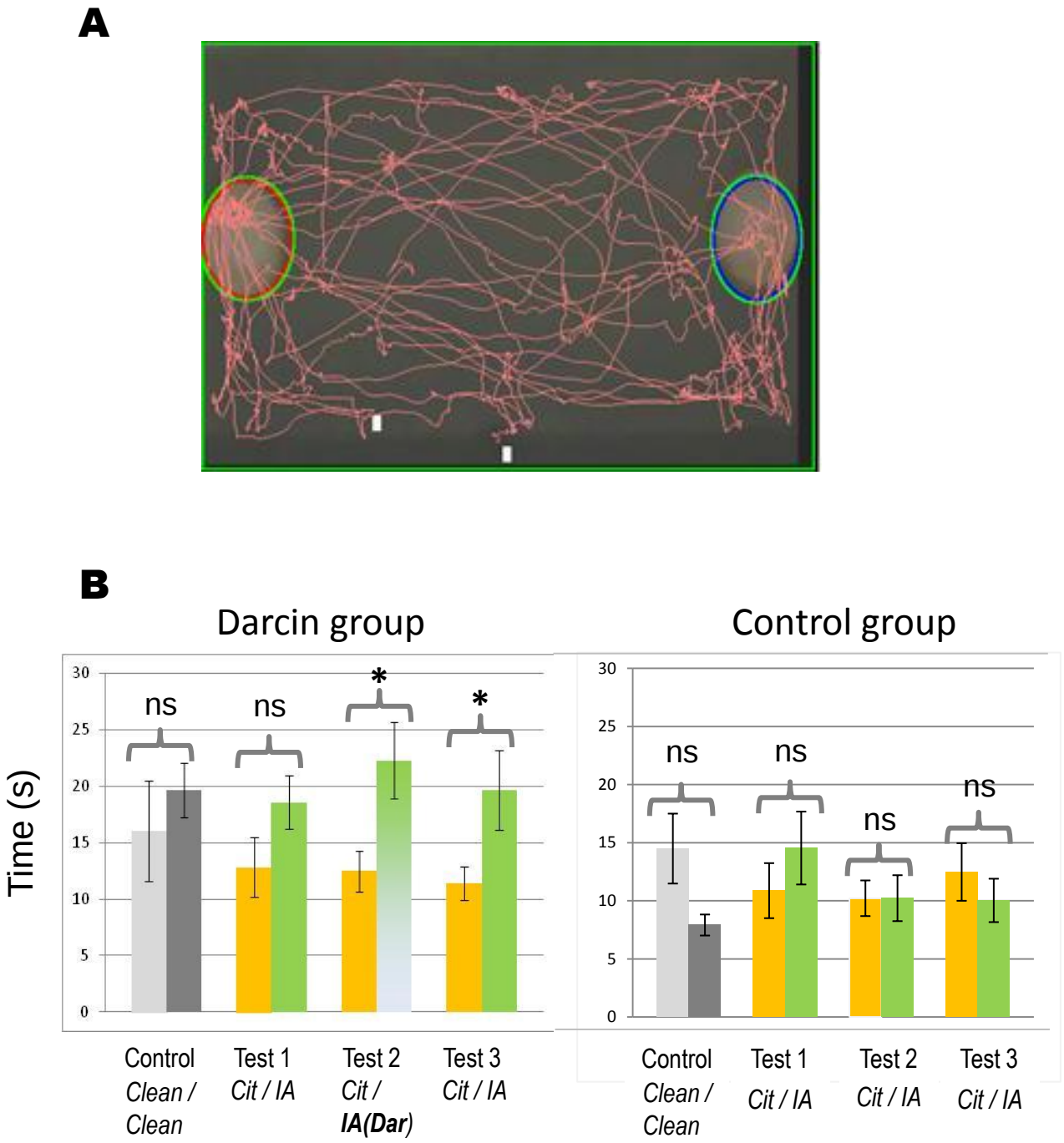
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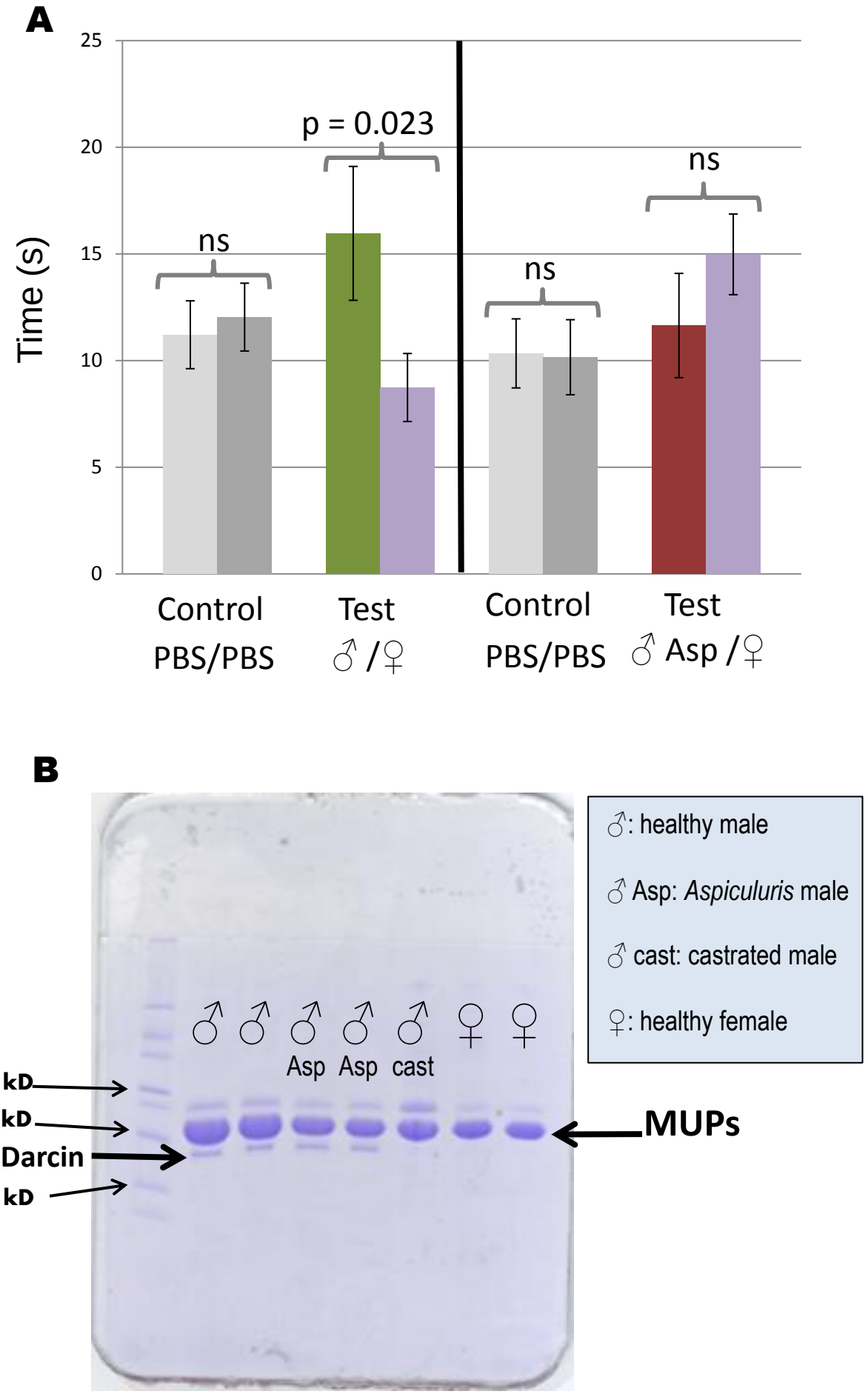
Figure 2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♂ Asp: Aspiculuris male
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