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Dissertação
Mestrado em Biologia Evolutiva e do Desenvolvimento

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ABSTRACT

Mate choice is an evolutionary process with a profound impact in species morphology, behavioural displays and overall success. We are interested in understanding the proximate mechanisms underlying the assortative mate choice exhibited by *Mus musculus musculus* females when given a choice between a male of their own subspecies and a male from the closely related subspecies, *Mus musculus domesticus*. Previous results from our laboratory suggest that this assortative preference is modulated by early life experience. Because mice rely primarily on olfactory cues for communication, our hypothesis is that *M. m. musculus* females are using an olfactory imprinting process in early life to establish their mating preferences. To understand which cues are important for this learning, we manipulated different elements of the mice's social context within the first weeks of life, such as the presence of the father. We also used classical conditioning with artificial odours to alter the olfactory experience of *M. m. musculus* females during post-natal development. We found the father's presence during *M. m. musculus* female's upbringing to be irrelevant in the establishment of the female's assortative preferences. Moreover, the early olfactory experience also seems to have no influence in the establishment of these preferences.

Key words: mate choice; assortative mating; olfaction; imprinting

RESUMO

Darwin baseou a sua teoria da evolução em duas formas de selecção. A primeira, selecção natural, refere-se à sobrevivência das espécies melhor adaptadas (Darwin, 1859). A segunda refere-se à luta para encontrar parceiros sexuais, sendo conhecida como selecção sexual (Darwin, 1871). Esta pode ser intrasexual, onde indivíduos de um sexo (normalmente os machos) lutam para ter acesso a parceiros sexuais, ou intersexual, onde as preferências de acasalamento das fêmeas, combinadas com competição entre os machos para atrair as fêmeas, determinam o sucesso de cada indivíduo.

Zonas híbridas são locais ideias para estudar selecção sexual e o seu papel na manutenção da identidade das espécies. Acasalamento selectivo, um tipo particular de selecção sexual onde os indivíduos preferem acasalar com parceiros que são fenotipicamente semelhantes, é um tipo comum de barreira pré-zigótica em zonas híbridas (Majerus, 1986; Shurtliff, 2011). Em mamíferos, uma das primeiras zonas híbridas a estudada foi a do murganho (*Mus musculus*), ocupada por duas subespécies, *Mus musculus musculus* e *Mus musculus domesticus*. Estas duas subespécies têm origem numa população ancestral do continente Indiano (Boursot, 1993) que divergiu em alopatria acompanhando a migração do homem, o desenvolvimento agrícola e a abertura de novos nichos ecológicos (Auffray, 1990). A subespécie *musculus* colonizou a Europa Oriental e o Norte Asiático, enquanto a subespécie *domesticus* ocupou a baía do Mediterrâneo e a Europa Ocidental, o que levou a um contacto secundário e à formação de uma zona híbrida que vai desde a Dinamarca até à Bulgária (Boursot, 1993).

Pensa-se que várias barreiras pré e pós-zigóticas contribuem para o baixo fluxo genético entre estas duas subespécies, uma delas sendo a forte selecção contra híbridos, evidenciada pela esterilidade (Forejt, 1996), aumento da carga parasítica (Moulia, 1991, 1993) e reduzida introgressão nos genes localizados nos cromossomas sexuais, dos híbridos. Para além da selecção contra os híbridos, as fêmeas da subespécie *musculus* exibem uma forte preferência sexual por machos da sua própria subespécie (Baudoin, 1998). Tal como em vários roedores, existem evidências de que as fêmeas *musculus* utilizam informação da urina e saliva dos machos para discriminar entre diferentes subespécies (Laukaitis, 1997; Ganem, 2001) e assim escolher um parceiro sexual. Esta preferência sexual parece ter evoluído no seio da zona híbrida e não durante a divergência em alopatria das duas subespécies, uma vez que a preferência sexual das fêmeas *musculus* é significativamente mais forte na zona de contacto entre as duas subespécies, do que em populações alopátricas (Smadja & Ganem, 2005).

No nosso laboratório, estamos interessados em perceber os mecanismos subjacentes às preferências sexuais das fêmeas *musculus*, especificamente, como é que a informação de múltiplos machos é processada para além da periferia, e também como é que experiência prévia e o estado interno das fêmeas influencia a sua decisão. Para responder a estas questões, nós estabelecemos um paradigma comportamental que simula a escolha natural das fêmeas na natureza entre os machos *musculus* e *domesticus* (Lima, 2013). Com este paradigma mostrámos que somos capazes de reproduzir, em condições controladas de laboratório, a preferência clássica exibida pelas fêmeas *musculus* por machos da sua própria subespécie. Esta preferência é evidenciada tanto em situações de contacto total, em que é permitido às fêmeas escolher com que machos preferem acasalar, como em situações de contacto limitado, onde apenas contacto entre os focinhos do macho e da fêmea é permitido.

Outro conjunto de experiências no nosso laboratório mostrou que as preferências sexuais das fêmeas *musculus* são altamente dependentes do ambiente em que estas fêmeas são criadas, uma vez que fêmeas criadas numa família da subespécie *domesticus* perdem a sua preferência natural por machos *musculus* (Lima, não publicado). O sistema olfactivo, a principal forma de comunicação em roedores, sobretudo em recém-nascidos, é provavelmente muito importante neste processo. Com base nestes resultados desenvolvemos uma hipótese de que as fêmeas estariam a aprender através dos cheiros presentes no ambiente pós-natal quais os machos com que deve acasalar em idade adulta. Para isso, manipulámos vários elementos do contexto social das fêmeas durante as suas primeiras semanas de vida, como por exemplo a presença do pai. Empregámos também técnicas de condicionamento olfativo, utilizando odores artificias para alterar a experiência olfactiva das fêmeas *musculus* durante as suas primeiras semanas de vida.

Vários elementos do ambiente pós-natal podem fornecer informação às fêmeas sobre com que machos acasalar em idade adulta. Resultados deste projecto mostram que a presença do pai no ambiente pós-natal não é necessária para que as fêmeas aprendam com que machos devem acasalar. Tanto fêmeas criadas com ou sem pai mostram uma forte preferência por machos da subespécie *musculus*. Estes resultados podem ser explicados se o pai não tiver qualquer papel na aprendizagem das preferências sexuais das fêmeas. Por outro lado, sendo esta preferência um elemento crucial para a manutenção do isolamento entre as duas subespécies, é possível que a aprendizagem seja de tal forma robusta que mesmo que as fêmeas utilizem informação proveniente do pai, consigam na sua ausência utilizar outras fontes de informação, como a mãe ou os irmãos.

Para investigar os efeitos da experiência olfactiva das fêmeas *musculus* na aprendizagem das suas preferências sexuais, utilizámos odores artificiais e condicionamento clássico para manipular a experiência pós-natal das fêmeas.

Começámos por utilizar citronela, um odor a limão, para alterar o cheiro do ninho durante o desenvolvimento das fêmeas. A nossa previsão era que as fêmeas expostas a citronela mostrassem uma preferência por este odor em idade adulta. No entanto, não encontrámos diferenças significativas no comportamento das fêmeas condicionadas e não-condicionadas com citronela. Na realidade, os primeiros conjuntos de experiências sugeriram que este odor poderia ser aversivo para as fêmeas, algo que viemos mais tarde a confirmar.

Uma vez que as experiências com citronela não nos permitiram testar a nossa hipótese acerca da importância das experiência olfactivas no desenvolvimento das preferências sexuais de fêmeas *musculus*, decidimos alterar a sua experiência olfactiva alterando o cheiro, não do ninho, mas da mãe. Para isso utilizámos outro par de odores, alho e baunilha, que foram pintados dia sim, dia não nos mamilos e área genital das mães. Começámos por testar o comportamento de fêmeas *musculus*, não condicionadas, em relação a estes dois odores, sem qualquer macho. Descobrimos que as fêmeas *musculus* têm uma aversão ao odor de alho e exibem uma resposta de neutralidade, ou ligeira atracção, para a baunilha. Em fêmeas criadas por mães que cheiravam a baunilha, não encontrámos um efeito significativo da nossa manipulação, uma vez que tanto fêmeas criadas com mães não manipuladas como fêmeas criadas com mães pintadas com baunilha, não mostram uma maior atracção por machos perfumados com baunilha. Estes resultados mostram que o nosso protocolo de condicionamento foi ineficaz em alterar as preferências sexuais das fêmeas.

Em conclusão, este estudo trouxe mais informação sobre o papel de experiência prévia na escolha de um parceiro sexual. Testámos diferentes protocolos de condicionamento olfactivo e avaliámos a importância do pai no desenvolvimento das preferências sexuais de fêmeas *musculus*. Os resultados aqui apresentados levantam também importantes questões que será importante responder no futuro. Isto permitir-nos-á aprender mais acerca dos mecanismos neuronais por detrás das preferências das fêmeas *musculus*.

Palavras-chave: Selecção sexual; zona híbrida; acasalamento selectivo; olfacção

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INTRODUCTION

Darwin based is theory of evolution on two different types of selection. The first, natural selection, described the struggle for existence and survival of the fittest (Darwin, 1859). The second referred to the struggle to secure mating partners, which became known as sexual selection (Darwin, 1871). The latter can either be intrasexual, where individuals of one sex (usually the males) compete for access to breeding partners, or intersexual, where female's mating preferences combined with competition between males to attract females, determines each male's success.

Contact zones between closely related or sibling species are ideal settings to study sexual selection and its role in maintaining species identity. Assortative mating, a particular type of sexual selection where individuals prefer to mate with partners that are phenotypically similar to them, is one of the commonly observed pre-zygotic barriers at such contact zones (Majerus, 1986; Shurtliff, 2011).In the threespine sticklebacks (*Gasterosteus aculeatus*), marine populations continuously give rise to numerous independent freshwater populations. Although these populations are sympatric (share the same habitat), they exhibit pre-mating isolation and low levels of introgression (Hagen, 1967). This is partly due to the fact that the anadromous fish (fish born in fresh water, that spend most of their life in the sea and return to fresh water to spawn) have larger body sizes when compared to the freshwater sticklebacks, and the probability of mating between the two populations is negatively correlated with the difference in body size (McKinnon, 2004). In this system, size-based assortative mating trough mating preferences for individuals with similar body size seems to be an important mechanism in keeping both populations isolated, although other traits can also be involved (McPhail, 1984; Braithwaite 2006).

In mammals, one of the first contact zones to be subjected to rigorous studies was that of the European house mouse (*Mus musculus*) subspecies, *Mus musculus musculus* and *Mus musculus domesticus*. These two subspecies originated from an ancestral population in the Northern Indian subcontinent (Boursot, 1993) and diverged in allopatry following human migration, agricultural development and the opening of new ecological niches (Auffray, 1990). The *musculus* subspecies colonized Eastern Europe and Northern Asia whilst the *domesticus* occupied the Mediterranean basin and Western Europe, which led to a secondary contact in Central Europe and the formation of a narrow (30 to 40 km) hybrid zone that currently spans for Denmark to Bulgaria (Boursot, 1993).

Several pre and post-zygotic mechanisms are thought to contribute to the low gene flow across these two subspecies, one of them being a strong selection against hybrids, evidenced by hybrid sterility (Forejt, 1996), increased parasitic load (Moulia, 1991; Moulia, 1993) and reduced introgression of genes located in

sex chromosomes (Dod, 1993). Aside from hybrid counterselection, *musculus* females exhibit a strong assortative mate preference for males of their own subspecies (Baudoin 1998); (Ganem 2005). Like most rodents, there is evidence that *musculus* females are using chemical cues in the male's urine and saliva to discriminate between different subspecies (Laukaitis 2007);(Ganem 2001) and choose a male sexual partner. This assortative preference appears to have evolved in the hybrid contact zone itself and not during the allopatric divergence, since *musculus* females preference is significantly stronger in populations within the contact zone compared to allopatric populations (Ganem 2005).

In our laboratory we are interested in understanding the mechanisms underlying the assortative mate preference of *musculus* females, particularly how multiple male signals are evaluated beyond peripheral processing and how the female's prior experience and internal state can influence mating decisions. To address these issues, we established a behavioural paradigm that mimics the natural *musculus* versus *domesticus* choice of males that *musculus* females have to perform in the wild (Lima 2013). We have shown that we are able to replicate, in laboratory controlled conditions, the classical homosubspecific mate preference of *musculus* females. Moreover, *musculus* females display this assortative preference both in limited contact situations, where only nose to nose contact between male and female is allowed, as well as in full mating situations.

Choosiness is expected to evolve in contact zones (Saether 1999). Here, a high capacity of discrimination and strong preferences for traits that distinguish between species are essential to avoid hybridization. How this choosiness evolves, both at a proximal and behavioural level, is quite a more difficult question to answer. Usually, and although somewhat meaningless, behaviours tend be classified as either innate or learned. For *musculus* females the same type of question can be made: Are the *musculus* female's learning from their lifetime experience which males they should mate with, or are they genetically hardwired to choose males of their own subspecies?

In a recent set of experiments, our lab has shown that *musculus* female's assortative preference can be altered by the social experience during their upbringing. We have shown that if *musculus* newborn females are fostered to a *domesticus* family, their preference for *musculus* males is disrupted (Zinck and Lima, unpublished). Instead, these females show no preference for either *musculus* or *domesticus* males. These results suggest that learning likely plays an important role in determining the mating preferences of *musculus* females. However, this does not necessarily rule out the involvement of a genetically determined component in the establishment of these preferences.

In many species mating preferences are learned throughout life, and when this learning takes place during an early stage of development, it is referred to as sexual imprinting (Immelmann 1972). This requires an irreversible learning from social interaction with a population of individuals, usually the parents or siblings,

known as the “imprinting set” (Servedio 2008), during a specific window of time called the critical period (Sullivan 2004). This allows animals to learn their species phenotype and recognize conspecific mates. Early work on sexual imprinting focused almost exclusively in studying song learning in precocial birds (Tamura, 1964), although recent studies have been made in other groups of vertebrates, such as fish (Kozak, 2011), amphibians (Ogurtsov, 2004) and, to a lesser extent, mammals (Kendrick, 1998). Amongst the first studies were the ones made by Immelmann (1969), who showed that male zebra finches (*Taeniopygia guttata*) prefer to court females of the species in which they were fostered, instead of their own species. An even more extreme case was described using eggs of great tits (*Parus major*) that were fostered in nests of blue tits (*Cyanistes caeruleus*). In this case, not only the adopted great tits preferred to mate with blue tits, but they also copied their song, developed a similar alarm call and copied their foraging niches (Slagsvold, 2007). This shows how the relationship between infants and tutors during early life can influence not only mating preferences, but several other aspects of adult social behaviour (Immelmann, 1975).

Overall, imprinting has been shown to play an important role in different evolutionary processes, such as speciation (Grant, 1997), interspecific brood parasitism (Payne, 2000) and sexual selection (Andersson, 1994). Song learning in two species of Darwin’s finches (*Geospiza fortis* and *Geospiza scandens*) is one of the best examples of how learning can act as a powerful pre-mating barrier. Experiments in the field demonstrated that when a different species of finch (*Geospiza magnirostris*), which sang in the same frequency as *G. fortis* and *G. scandens*, invaded their habitat the temporal features of the song of these two species started diverging. These differences arose as a bias during the song imprinting process, since both *G. fortis* and *G. scandens* sons started singing faster songs compared to their fathers (Grant, 2010). This peak shift mechanism could have important implications in several other speciation events, especially those involving reproductive character displacement. For instance, in sympatric species sexual imprinting can help discriminate between very similar phenotypes and lead to a skewed generalization that prefers stimuli even more divergent that the one being imprinted on. Likewise, if the imprinted phenotype is similar to the phenotype of the own individual this leads to individuals preferring phenotypically similar mates. This is one of the ways through which sexual imprinting can lead to assortative mating and conspecific mating preferences (Verzijden, 2012), which could be an important mechanism behind the evolution of the assortative preference shown by *musculus* females.

In rodents olfaction is a critical sensory modality for survival, allowing individuals to gather information about resources, avoid predators, establish social hierarchies and find mating partners (Colwell, 2001; Brennan, 2004; Hurst, 2009). Mice are no exception and the ability to distinguish the molecular cues of other individuals plays a crucial role in their social interaction. Here, odours convey

information about themselves but also allow the recognition of conspecifics and the assessment of potential mates (Ganem, 2001).

The olfactory system of mice is composed of two different subsystems, the main and accessory olfactory systems (MOS and AOS, respectively), which are extremely intermingled and differ both in anatomy, function and central projections (Hurst, 2008). A fundamental feature of rodent's olfactory system is its diversity. In mammals, approximately one thousand odour receptor (OR) genes are solely dedicated to olfaction (Zufall, 2012). This is an even more impressive feature if we take into account that each olfactory sensory neuron (OSN) only expresses one type of odour receptor. Besides odour detection, this allows for an incredible power of olfactory discrimination and recognition. Interestingly, mice are even able to distinguish between enantiomeric odours, pairs of odorous with identical chemical and physical properties with exception to their optical activity (Shepherd, 2007).

Three classes of non-volatile proteins and small peptides secreted by male mice are thought to be essential for the female's assessment of different males as potential mates. Major urinary proteins (MUPs) seem to be especially important for the discrimination of individual scent marks, since the MUP signature profile of each male in the wild is different (Held, 1985). Although major histocompatibility complex (MHC) peptides also exhibit a high degree of polymorphism, females don't seem to rely on them to distinguish between males (Singer, 1997). Instead, the MHC appears to be more relevant for the recognition of related individuals. By comparing the male's MHC haplotype to their own, females are able to avoid mating with kin individuals, therefore promoting heterozigosity at MHC loci and increasing resistance to pathogens in the offspring (Penn, 2002). The expression profile of exocrine-gland secreting peptides (ESPs) in male's tears also appears to convey individual variability, although the role of these proteins in the social and sexual behaviour of mice is not very clear yet (Touhara, 2005). Overall, these three gene families all seem to participate in the female's olfactory assessment of a male's identify and evaluation of his attractiveness as a potential mating partner.

Several studies show that olfactory preferences in rodents could be highly influenced by early life odour experience (Keverne, 1990; Sullivan, 2005). Indeed, odours present in the environment during mice post-natal development seem to be preferred over unfamiliar odours in adulthood (Blass, 1986; Sullivan, 1994). Learning the odours present in the rearing environment and remembering them as an adult could be an important mechanism for female's to ensure mating happens with conspecifics. Cross-fostering experiments where adopted mice show an attraction towards the odours of the foster species, indicate that species-specific odour cues are learned during early life (Boyse, 1988; Penn, 2002). The adoption of mice in rat families, which results in a preference for rats in adopted mice adulthood, while rats are natural mice predators, is one of the most amazing

examples of this phenomenon (Denenberg,1964). However, this type of experiments is also pervious to several confounding variables that could influence mice's olfactory preferences, such as parental care, sibling sociality and other ecological factors.

Another common approach involves conditioning mice with exposure to artificial odours during early life (Bouslama, 2005). These odours are usually added to either the nesting environment or directly to one of the parents, thus ensuring a more direct manipulation of the pup's olfactory experience and also eliminating some of the caveats associated with the cross-fostering experiments. Overall, early life exposure to a neutral artificial odour seems to increase the value of that odour in adulthood to the point where conditioned mice show an attraction to individuals scented with that odour (Mainardi, 1965). In one experiment, Blass (1986) reared male rats with damns whose nipples and vagina were painted with a lemon scent. He showed that in adulthood the conditioned males ejaculated faster when paired with lemon-scented females than with females non-scented ones. These studies demonstrate how initially neutral and biologically non-relevant stimuli can influence adult sexual behaviour. Moreover, they suggest that several biologically relevant odours are initially neutral and gain importance during social interaction in pre and post-natal development (Stowers, 2012).

In our opinion, there seems to be a clear role of early life experience in determining female mice mating behaviour and olfaction likely plays a key part in this process. Therefore, we hypothesize that an olfactory sexual imprinting mechanism could help explain the assortative mating preference of *musculus* females in the *Mus musculus* contact zone. Since olfaction is a major communication highway in mice, we reason that *musculus* females might be learning from the odour of the parents, possibly the father, which males to mate with later in life. To test this hypothesis we performed several experiments where we manipulated the early experience of the females, either by manipulation of the nest composition (removing the father) or the nest odour (conditioning with artificial odours), and tested the effect of these manipulations on the assortative mate choice of *musculus* females later in life. Understanding how early olfactory experience influences adult mating behaviour will be the first step in the future goal of trying to unravel the neuronal circuitry underlying this early learning mechanism.

MATERIALS & METHODS

1) Animals

As representatives of the *Mus musculus musculus* subspecies we used the PWD/PhJ and PWK/PhJ strains, ordered from The Jackson Laboratory and derived originally from animals trapped in Czech Republic in 1972 and later inbred trough sister-brother crossing in the laboratory (Forejt, 2000). As representative of the *Mus musculus domesticus* subspecies we used the classical laboratory strain C57BL/6J, also ordered from The Jackson Laboratory. All animals were weaned at 21 days of age and housed in same-sex groups of two to six animals in standard cages (1284L, Techniplast, 365 x 207 x 140 mm). Food and water were provided *ad libitum*. Animals were maintained in a 12:12 light/dark cycle with light onset at 0800 and all behavioural testing was performed at least 2 hours after light onset. Cage changing was performed once per week for *domesticus* animals and once every other week for *musculus*. To enhance female receptivity and ensure proper olfactory development, all *musculus* females were exposed to male soiled bedding or 10 µL of male urine in alternating weeks. Both the soiled bedding and the urine were a mixture of equal volume from PWK/PhJ, PWD/PhJ and C57BL/6J males. Animals were sacrificed by CO_2 asphyxiation followed by cervical displacement at the end of each set of experiments.

2) Early life manipulation 1: Removing the musculus father from the rearing environment

Previous results from our laboratory have shown that PWD/PhJ newborn females fostered in a BALB/c family, another *domesticus* laboratory strain, don't show the natural homosubspecific preference towards *musculus* males. These results suggest that *musculus* females are using an early life imprinting mechanism to learn which males to mate with in adulthood. In this process, visual, auditory and olfactory cues are learned from the imprinting set, which is usually made up of the parents and/or siblings. For *musculus* females, the father is the only adult male that they encounter during their entire upbringing, which makes him a good candidate to be mediating this learning process. Furthermore, other studies have shown that *domesticus* females reared only by their mother show different male preferences than those reared by both sets of parents (Mainardi, 1964). Thus, we believe the presence of the father during early life to be an essential element in the establishment of the assortative preferences of *musculus* females.

Therefore, we decided to raise PWD/PhJ litters only by their mothers and, as adults, evaluate their mating preferences compared to normally reared females. The PWD/PhJ father was removed from the breeding at least 5 days before the mothers gave birth, meaning that the *musculus* newborn females never came into contact with an adult *musculus* male before behavioural testing.

3) Early life manipulation 2: Olfactory conditioning with citronellal

In rodents, olfaction mediates both learned and innate behaviours, from food scavenging, to predator avoidance and searching for sexual partners. Our hypothesis is that *musculus* females learn the odours present in the early life environment, assign positive value to these cues and remember them in adulthood, when choosing a male sexual partner. To address this hypothesis, we used a classical conditioning protocol to increase the value of neutral, artificial odours in female adulthood by exposing them to these cues during early life. Artificial odours have already been used successfully to do classical conditioning in newborn mice (Bouslama, 2005) and many studies in mammals have already used pup exposure to odors to study subsequent odor or social preferences (Blass, 1986; Shah, 2002; Mateo, 2009). Using artificial odours to condition *musculus* females allowed us to, not only to reduce the immense variability of olfactory stimuli in the female's rearing environment to a single, very salient, odour, but also to use this odour to manipulate male value in adulthood.

We used citronellal (Sigma-Aldrich, 27470, St. Louis, MO), a lemon-scent, which had been previously described to be innately neutral for *Mus musculus* neonatal mice (Armstrong, 2006). Therefore, neonatal PWD/PhJ *musculus* litters were exposed to citronellal between birth and weaning. The citronellal stock solution was diluted in mineral oil (Sigma-Aldrich, M31516, St. Louis, MO) to a volume/volume dilution of 166 µl/ml. 100 µl of the diluted solution were gently pipetted everyday around the litter nest, from postnatal day (PN) 0 until PN21 or from PN0 to PN10. After weaning, the females were housed together and never came into contact with citronellal again. Behavioural testing was performed when these females reached adulthood (3 to 4 months old). The stimulus males were painted using a brush soaked in the citronellal solution, once before every test session.

4) Early life manipulation 3: Olfactory conditioning by alteration of dams scent

Neonatal rodent's ability to quickly locate and attach to the mother's nipple is fundamental for its survival, and this behaviour is heavily dependent on olfaction (Hongo, 2000). Moreover, the initial experience with milk has been shown to reinforce components of early suckling behaviour (Cheslock, 2004). This strongly suggests that the olfactory stimuli associated to the mother's nipple is of the most important signals mice need to learn and recognize, which gives us an excellent opportunity to manipulate neonatal mice olfactory experience. Other studies in rodents have shown that alteration of the mother's nipple and genital odours lead to an increased preference towards these odours in litters reared with by such mothers (Blass, 1986; Mainardi, 1965). Therefore, we decided to manipulate newborn *musculus* female's early olfactory experience by altering the odours associated to the mother.

PWD/PhJ litters were raised by dams whose nipple and vagina were altered with garlic or vanilla odorants. This particular pair of odours was chosen because a recent study found garlic and vanilla odours to be neutral for neonatal mice (Stowers, 2012). Garlic solutions were prepared by diluting a garlic oil solution (Sigma-Aldrich, W250309, St. Louis, MO) to a 2.13% concentration, using water and 0.14% Emplex (Caravan Ingredients, Lenexa, KS), an emulsifying agent used to help blend the garlic oil in water. Vanilla solutions were prepared by diluting 1.52g of vanillin (Sigma-Aldrich, V1104, St. Louis, MO) in 100 ml of water and heating the mixture until the vanilla was completely dissolved. Controls consisted of PWD/PhJ litters raised by damns whose nipple and vagina were painted with water.

The vagina and nipples of the dams were painted only every other day, using cotton tips, to reduce the stress levels of both mothers and pups. To make sure that the mothers were already scented during the first suckling event of the newborn mice, they were painted at least 1 day before giving birth. After PN21, the female pups were weaned, never came into contact with neither garlic nor vanilla again and, when they reached adulthood, were tested for male preference. Stimulus males were painted with either the garlic or vanilla diluted solutions once before every trial using cotton tips.

5) Behavioural testing

To investigate the *musculus* female's mate preference we used a limited-contact paradigm (Social Preference Test, SPT) previously developed and validated in our laboratory (Lima, 2013). The behavioural apparatus is made up of three transparent acrylic boxes (200 x 150 x 150 mm), where a central box is connected to two side boxes by acrylic tubing with 30 mm diameter and 50 mm long. In each side box there's a 1 mm. thick polyvinyl chloride (PVC) partition with four holes of 8 mm. in diameter at its centre, 20 to 40 mm from the floor, which allows nose to nose contact between the male and the female. Before each trial the floor of the three boxes was covered with clean bedding, and one disposable hut plus two food pellets were placed inside the central box, to reinforce the neutral state of this box. After each trial the entire apparatus was washed with Virkon® and air-dried.

In each set of experiments, the *musculus* females were habituated to the behavioural apparatus for 20 minutes during 3 days. In the habituation trials the females were placed inside the central box and allowed to explore the entire apparatus, without males, for 15 minutes. When those 15 minutes were over, and the females returned to the central box, the connections to the side boxes were closed, forcing the females to stay another 5 minutes inside the central box, before being taken back to their home cage. Only females that visited both sides of the apparatus during each of the 3 days of habituation, and showed no bias towards any of them, were analysed. After the 3 days of habituation, each female was tested twice, once 24 hours after the last day of habituation, and a second

time 48 hours after the first test day. On the test days, the experimental protocol was the same, however during the 5 minutes in which the females were forced to stay inside the central box, two males were randomly assigned to the side boxes. When the 5 minute period was over, the connections of the central box to the side boxes were re-opened and the females allowed to explore the entire apparatus for another 15 minutes. When the test was over, and before returning the females to their home cage, vaginal smears were performed to determine the estrous state of the females. A cytological staining protocol was then used to reveal the cellular composition of the vaginal smears. Non-receptive females, in diestrous, were identified by smears containing leucocytes, while females in pro-estrous/estrous or estrous were characterized by smears with a mixture of nucleated epithelial and anucleated cornified cells or only cornified cells, respectively. In all the behavioural experiments, the choosing female was always a PWD/PhJ *musculus* with 3 to 4 months of age. The stimulus males were always PWK/PhJ *musculus* and C57BL/6J *domesticus* with at least 2 months and no more than 1 month age difference between them. We used PWD/PhJ *musculus* females as choosers because PWK/PhJ adult females show higher levels of stress when handled, as well as other behavioural impairments. Additionally, the *musculus* stimulus males were always from strain PWK/PhJ to avoid inbreeding/familiarity confounding effects.

The stimulus males for each set of experiments were isolated in stand-alone cages (1145T, Techniplast, 369 x 156 x 132 mm) 2 weeks prior to the females' behavioural testing to control for social ranking effects. During these 2 weeks, they were paired twice with ovariectomized C57BL/6J females for a maximum of one hour to ensure that they were properly engaged and sexually motivated to interact with the females during the behavioural trials. Only the males that showed consistent sexual motivation, during both training sessions were used as stimulus males. Each pair of males was used a maximum of 2 times in each day of behavioural testing.

6) Behavioural analysis

Mice behaviour was recorded using Sony cameras (HDR-HC7E) connected to a computer running Virtual Dub software to acquire frame by frame images (30 fps). All habituation and test trials for each female were recorded. Analysis of the behaviour was performed semi-automatically, using BONSAI software (Gonçalo and Adam, not published) and by defining Regions of Interest (ROI's) in the female's side of the male boxes. Female behaviour parameters analysed include: 1) time spent in each male box; 2) number entries in each male box and 3) number of re-entries in each male box (situations where the female re-entered the male box where she previously was). The preference score for each female was calculated as: (time spent with *musculus*) / ((time spent with *musculus*) + (time spent with *domesticus*)). BONSAI output files were analysed in MATLAB R2010b (version 7.11.1) to produce different types of graphs.

7) Statistical analysis

To investigate the influence of the male genotype and estrous state in the time spent and the number of visits to each male (*musculus* or *domesticus*) we used two-way ANOVA tests. Females in pro-estrous/estrous or estrous phase were treated as being receptive, and females in diestrous as being non-receptive. Data normality was tested beforehand, using Shapiro-Wilk tests. Otherwise, we used Non-parametric tests, which rely on no particular assumption. For paired samples we used Wilcoxon signed-rank tests. For independent samples we used Mann-Whitney tests and also Kruskall-Wallis for multiple pairwise comparisons, using a Bonferroni correction of *p-value*. P-value was calculated using an exact method and significance was accepted at $P < 0.05$. Data is always expressed as mean ± standard error (X ± SE). Statistical analyses was performed using Addinsoft XLSTAT-Pro software.

Results

Musculus females show an assortative preference for musculus males

We started by testing musculus female's preference between a *musculus* and a *domesticus* male using a social preference test (SPT) which only allows limited contact between males and females (only nose-nose contact) (Fig 1A) in order to reproduce data from our laboratory. Females were successfully habituated to the SPT box as they exhibited a decreased latency to enter either one of the side boxes along the habituation sessions (Fig 1B) suggesting a significant reduction of their stress level and increased willingness to explore the entire setup. As expected, in the first day of test, females tended to spend more time with *musculus* males than with the *domesticus* males (Fig 2A). Although not significant, the average time spent by females with each male suggests a preference for the *musculus* male. In the second test, all the females showed a preference for the *musculus* male (preference score above 0.5, Fig 2C) resulting in a significant and increased overall assortative preference (Fig 2A, C).

We also looked at the number of visits to each male box. In the first test, females tended to visit the *musculus* box more often, although there is no significant difference (Fig 2B). In the second test, the number of visits to the *musculus* male box is significantly higher than for the *domesticus* box, confirming the strong assortative preference of *musculus* females (Fig 2B).

Finally, we investigated if female's preference was dependent of their estrous state. There was only a main effect of male genotype in the second test, but no effect of the estrous state, in the total time spent with each male (Supplementary Fig. 1).

Overall, these results are in agreement with what had been previously shown in the laboratory, that *musculus* females exhibit an assortative preference for *musculus* over *domesticus* males, and that the estrous state of the female does not influence her choice in limited contact situations, where mating is not allowed. Interestingly, we also observed that the female's preference for *musculus* males increases from the first to the second test (Fig 2A and Fig 2B).

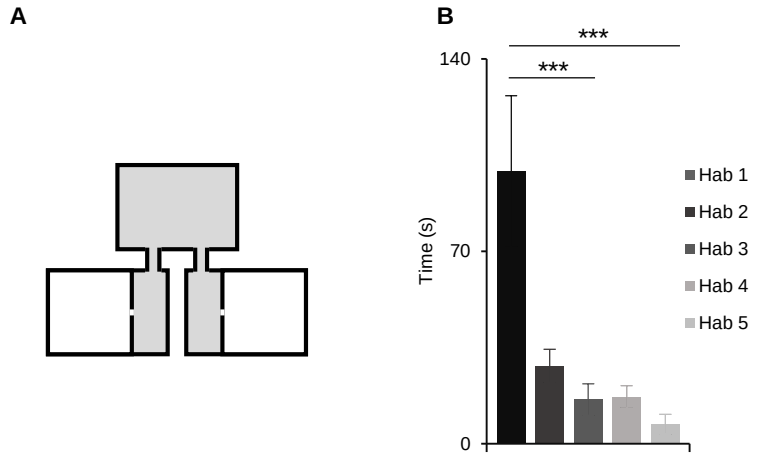

Figure 1. A) Schematic representation of the behavioural box used for Social Preference Test (SPT); B) Average latency of the first entry in seconds to one of the side boxes during the 15 minute habituations (Kruskall Wallis test, K = 18.8, N = 7, with a Bonferroni corrected significance level); ***means *P* < 0.001.

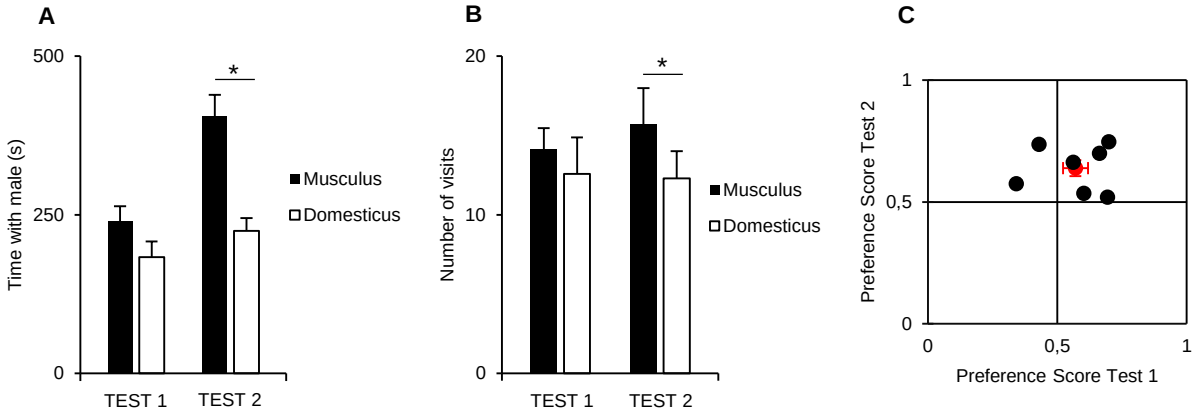

Figure 2. A) Time spent in seconds by *musculus* females with each male in the first (X ± SE *musculus* = 240.35 ± 25.07, N=7; X ± SE *domesticus* = 183.41 ± 26.67, N=7; Wilcoxon signed-rank test, V = 22, *P* = 0.219) and second test (X ± SE *musculus* = 404.76 ± 37.38, N=7; X ± SE *domesticus* = 224.58 ± 21.87, N=7; Wilcoxon signed-rank test, V = 28, *P* = 0.016). B) Visit number to each male by *musculus* female's in the first (X ± SE *musculus* = 14.14 ± 1.42, N=7; X ± SE *domesticus* = 12.57 ± 2.50, N=7; Wilcoxon signed-rank test, V=18, *P* = 0.498) and second test (X ± SE *musculus* = 15.71 ± 2.47, N=7; X ± SE *domesticus* = 12.29 ± 1.87, N=7; Wilcoxon signed-rank test, V= 27, *P* = 0.027). C) *Musculus* female's preference score for *musculus* males in the first and second test (X ± SE P.S. in the first test = 0.57 ± 0.05, N=7; X ± SE P.S. in the second test = 0.64 ± 0.03, N=7; Wilcoxon signed-rank test, V = 8, *P* = 0.375); * means P < 0.05; Black dots are individual data and red dot is the mean ± SE.

Early experience manipulation 1: Absence of the father during musculus female's upbringing has no effect on their assortative mate preference

To investigate if *musculus* females use a sexual imprinting mechanism based on the father to establish their adult mating preferences, we raised newborn females without a father from birth until weaning. We found that *musculus* females raised without their father showed a similar preference to those of normally reared females during both tests, in all measures analysed (time spent and visit number, Fig. 3 A - D). Although no significant statistical differences were found in the time spent and number of visits (except visit number in the test 1) for females reared without a father, the graphs show a clear trend for preference of *musculus* males. These results show that, although the social context of the nest was altered (by removal of the father), the characteristic assortative mate preference of *musculus* females, and the increase in preference with re-testing, were maintained.

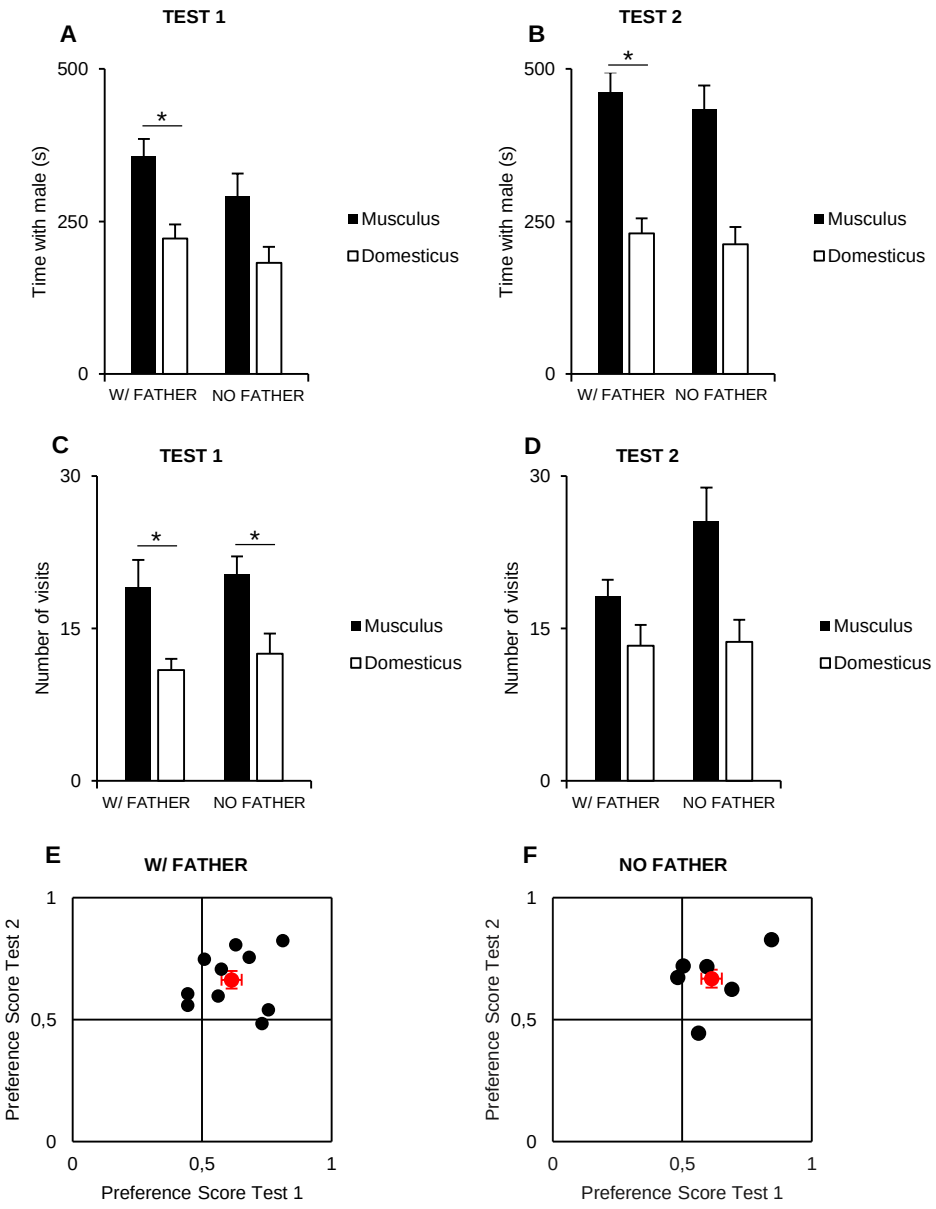

Figure 3. A) Time spent in seconds by *musculus* females with each male in the first test ("W/ FATHER": X ± SE *musculus* = 355.78 ± 31.11, N=10; X ± SE *domesticus* = 221.91 ± 24.12, N=10; Wilcoxon signed-rank test, V = 48, *P* = 0.037; "NO FATHER": X ± SE *musculus* = 291.36 ± 40.43, N=6; X ± SE *domesticus* = 181.80 ± 28.95, N=6; Wilcoxon signed-rank test, V = 19, *P* = 0.094). B) Time spent in seconds by *musculus* females with each male in the second test ("W/ FATHER": X ± SE *musculus* = 461.41 ± 34.68, N=10; X ± SE *domesticus* = 230.55 ± 25.63, N=10; Wilcoxon signed-rank test, V = 54, *P* = 0.004; "NO FATHER": X ± SE *musculus* = 433.97 ± 42.75, N=6; X ± SE *domesticus* = 212.62 ± 31.02, N=6; Wilcoxon signed-rank test, V = 20, *P* = 0.063). C) Visit number to each male by *musculus* female's in the first test ("W/ FATHER": X ± SE *musculus* = 19.10 ± 2.78, N=10; X ± SE *domesticus* = 10.90 ± 1.16, N=10; Wilcoxon signed-rank test, V = 47, *P* = 0.047; "NO FATHER": X ± SE *musculus* = 20.33 ± 1.91, N=6; X ± SE *domesticus* = 12.5 ± 2.17, N=6; Wilcoxon signed-rank test, V = 21, *P* = 0.027). D) Visit number to each male by *musculus* female's in the second test ("W/ FATHER": X ± SE *musculus* = 18.10 ± 1.76, N=10; X ± SE *domesticus* = 13.30 ± 2.14, N=10; Wilcoxon signed-rank test, V = 47, *P* = 0.053; "NO FATHER": X ± SE *musculus* = 25.50 ± 3.68, N=6; X ± SE *domesticus* = 13.67 ± 2.38, N=6; Wilcoxon signed-rank test, V = 2, *P* = 0.063). E) *Musculus* female's reared with a father preference score for *musculus* males in the first and second test (X ± SE P.S. in the first test = 0.61 ± 0.04, N=10; X ± SE P.S. in the second test = 0.66 ± 0.04, N=10; Wilcoxon signed-rank test, V = 18, *P* = 0.375). F) *Musculus* female's reared without a father preference score for *musculus* males in the first and second test (X ± SE P.S. in the first test = 0.61 ± 0.04, N=6; X ± SE P.S. in the second test = 0.67 ± 0.04, N=6; Wilcoxon signed-rank test, V = 6, *P* = 0.438).* means $P < 0.05$, ** means $P < 0.01$ Black dots are individual data and red dot is the mean ± SE.

Early experience manipulation 2: Environmental exposure to citronellal during early life doesn't influence mate choice in musculus females

As early life exposure to an artificial odour is supposed to increase its value in adulthood we conditioned newborn *musculus* females to citronellal during their first weeks of life and tested their preference between a *musculus* and a citronellal-scented *domesticus* male. Our hypothesis is that the citronellal scent would increase *domesticus* male value for females conditioned to this odour, and revert their natural preference for *musculus* males.

We found that, citronellal conditioned and non-conditioned *musculus* females, showed a similar behaviour regarding the time spent and the number of visits to each male, during both test (Fig. 4A - D). In test one as well as in test two, both groups of females exhibited a preference score for *musculus* males much higher than 0.5, which indicated a robust preference for the *musculus* males over the citronellal-scented *domesticus* (Fig. 4E, F). Moreover females exposed to citronellal from PN0 to PN10 (N = 14) and from PN0 to PN21 (N = 5) showed similar levels of preference (black dots and grey dots respectively, Fig. 4F).

Control experiments to further investigate the behaviour of both conditioned and non-conditioned females towards citronellal were performed in social and non-social contexts. We found that when females were given the choice between two *musculus* males, one of them being scented with citronellal, both conditioned and non-conditioned females spent significantly more time near the male without odour. Similarly, when the odour was presented on a paper filter, and males absent of the boxes, conditioned females spent more time in the box without odour (Supplementary Fig. 2).

Altogether these results suggest that citronellal is an aversive odour for adult *musculus* females, and/or that mice may avoid odorant places.

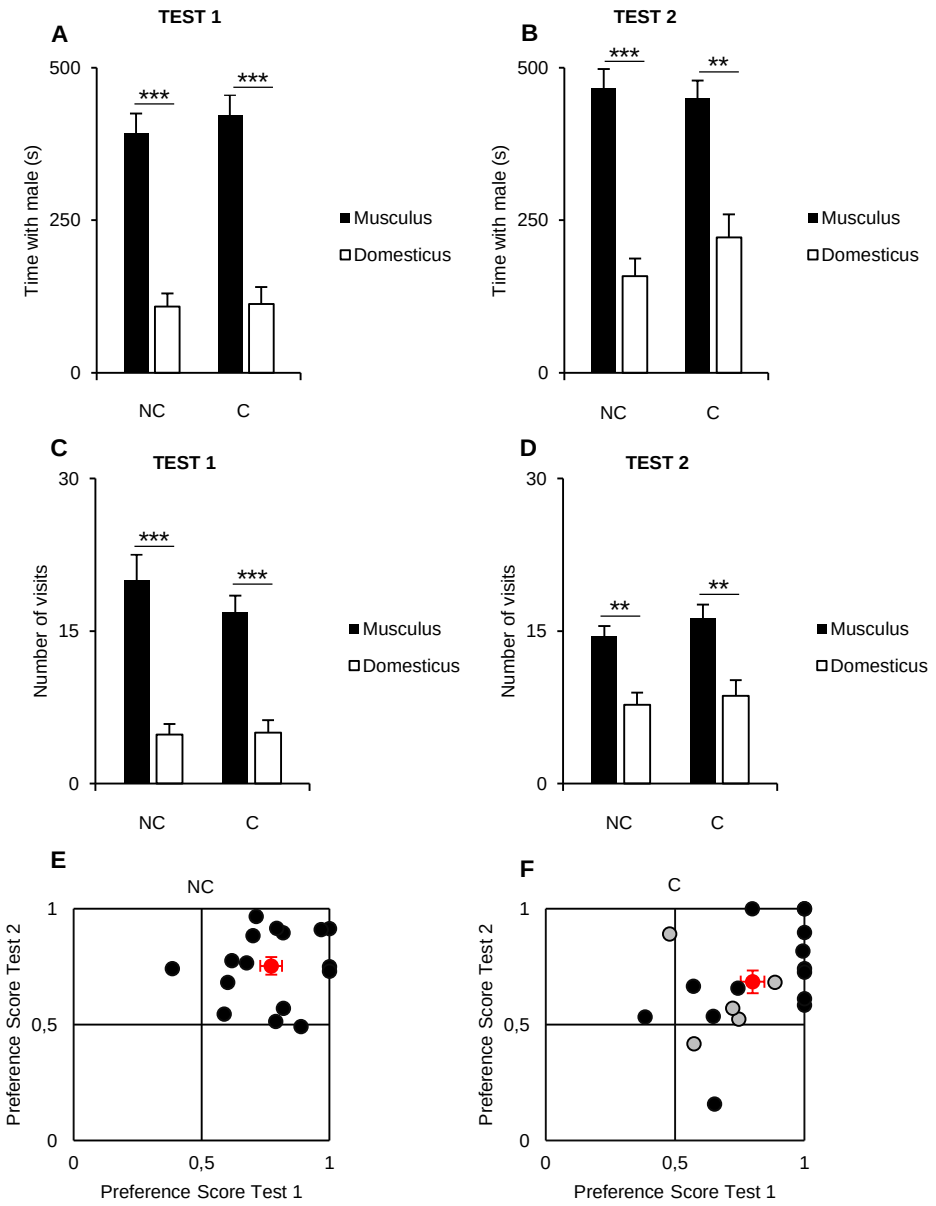

Figure 4. A) Time spent in seconds by *musculus* females with each male in the first test ("NC", Non-Conditioned: X ± SE *musculus* = 391.88 ± 34.14, N=16; X ± SE *domesticus* = 108.57 ± 21.84, N=16; Wilcoxon signed-rank test, V = 132, $P < 0.001$; "C", Conditioned: X ± SE *musculus* = 422.39 ± 33.74, N=19; X ± SE *domesticus* = 112.78 ± 28.53, N=19; Wilcoxon signed-rank test, V = 185, $P < 0.001$). B) Time spent in seconds by *musculus* females with each male in the second test ("NC", Non-Conditioned: X ± SE *musculus* = 466.67 ± 32.21, N=16; X ± SE *domesticus* = 158.28 ± 30.08, N=16; Wilcoxon signed-rank test, V = 135, $P < 0.001$; "C", Conditioned: X ± SE *musculus* = 449.80 ± 29.87, N=19; X ± SE *domesticus* = 221.62 ± 38.89, N=19; Wilcoxon signed-rank test, V = 168, $P = 0.002$). C) Visit number to each male by *musculus* female's in the first test ("NC", Non-Conditioned: X ± SE *musculus* = 20 ± 2.60, N=16; X ± SE *domesticus* = 4.81 ± 1.07, N=16; Wilcoxon signed-rank test, V = 120, $P = 0.001$; "C", Conditioned: X ± SE *musculus* = 16.84 ± 1.67, N=19; X ± SE *domesticus* = 5 ± 1.27, N=19; Wilcoxon signed-rank test, V = 179, $P = 0.001$). D) Visit number to each male by *musculus* female's in the second test ("NC", Non-Conditioned: X ± SE *musculus* = 14.5 ± 1.02, N=16; X ± SE *domesticus* = 7.75 ± 1.21, N=16; Wilcoxon signed-rank test, V = 122, $P = 0.006$; "C", Conditioned: X ± SE *musculus* = 16.32 ± 1.32, N=19; X ± SE *domesticus* = 8.63 ± 1.54, N=19; Wilcoxon signed-rank test, V = 149, $P = 0.006$). E) Non-conditioned *musculus* female's preference score for *musculus* males in the first and second test (X ± SE P.S. in the first test = 0.77 ± 0.04, N=16; X ± SE P.S. in the second test = 0.75 ± 0.04, N=16; Wilcoxon signed-rank test, V = 72, $P = 0.860$). F) Citronellal conditioned *musculus* female's preference score for *musculus* males in the first and second test (X ± SE P.S. in the first test = 0.80 ± 0.05, N=19; X ± SE P.S. in the second test = 0.68 ± 0.05, N=19; Wilcoxon signed-rank test, V = 135, $P = 0.031$); ** means $P < 0.01$, *** means $P \leq 0.001$; Black dots are individual data and red dot is the mean ± SE; grey dots are females conditioned from PN0 to PN21.

Early experience manipulation 3: Manipulation of dam scent does not impact the social preference of musculus females

To determine if garlic and vanilla odours could be used in the next set of experiments we started by testing the response of *musculus* female's (non-conditioned) to each of these odours, in non-social context. Females were presented with two paper filters with either garlic vs. water or vanilla vs. water. Our results showed that *musculus* females had a neutral, or slightly appetitive, response towards vanilla (Fig. 5A) and tended to avoid the garlic odour (Fig. 5B).

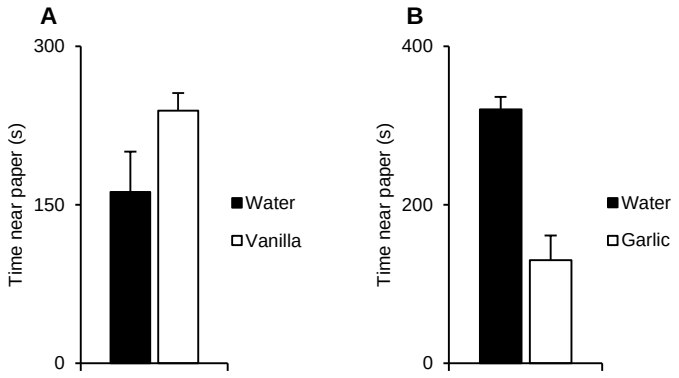

Figure 5 A) Time spent in seconds by *musculus* females near a vanilla or water paper filter (X ± SE water = 162.04 ± 38.13, N=4; X ± SE vanilla = 239.10 ± 16.60, N=4; Wilcoxon signed-rank test, V = 1, *P* = 0.250). B) Time spent in seconds by *musculus* females near a garlic or water paper filter (X ± SE water = 320.48 ± 15.58, N=4; X ± SE garlic= 129.96 ± 31.45, N=4; Wilcoxon signed-rank test, V = 10, *P* = 0.125).

We then tested the preference of *musculus* females raised by vanilla-scented dams when they were given a choice between a *musculus* male painted with vanilla and a *musculus* male painted with water. We found no significant differences in the behaviour of conditioned and non-conditioned females (time spent and visit number during both tests (Fig. 6A - D).

A significant difference in the preference score of females was found in the first test, during which conditioned females spent more time with the males without vanilla (X ± SE P.S. for water of non-conditioned females = 0.44 ± 0.05, N=5; X ± SE P.S. for water of conditioned females = 0.83 ± 0.12, N=3; Mann-Whitney test, U=0, *P* = 0.04) (Fig. 6E). This difference was likely due to the low number of conditioned females tested, since in the second test, usually more reliable, all females spent a similar amount of time with each male (Fig. 6B) and had similar preference scores (X ± SE P.S. for water of non-conditioned females = 0.51 ± 0.05, N=5; X ± SE P.S. for water of conditioned females = 0.57 ± 0.07, N=3; Mann-Whitney test, U=4, *P* = 0.40) (Fig. 6E). These results suggest that the particular type of manipulation of *musculus* female's early life olfactory experience that we performed does not influence adult social preference.

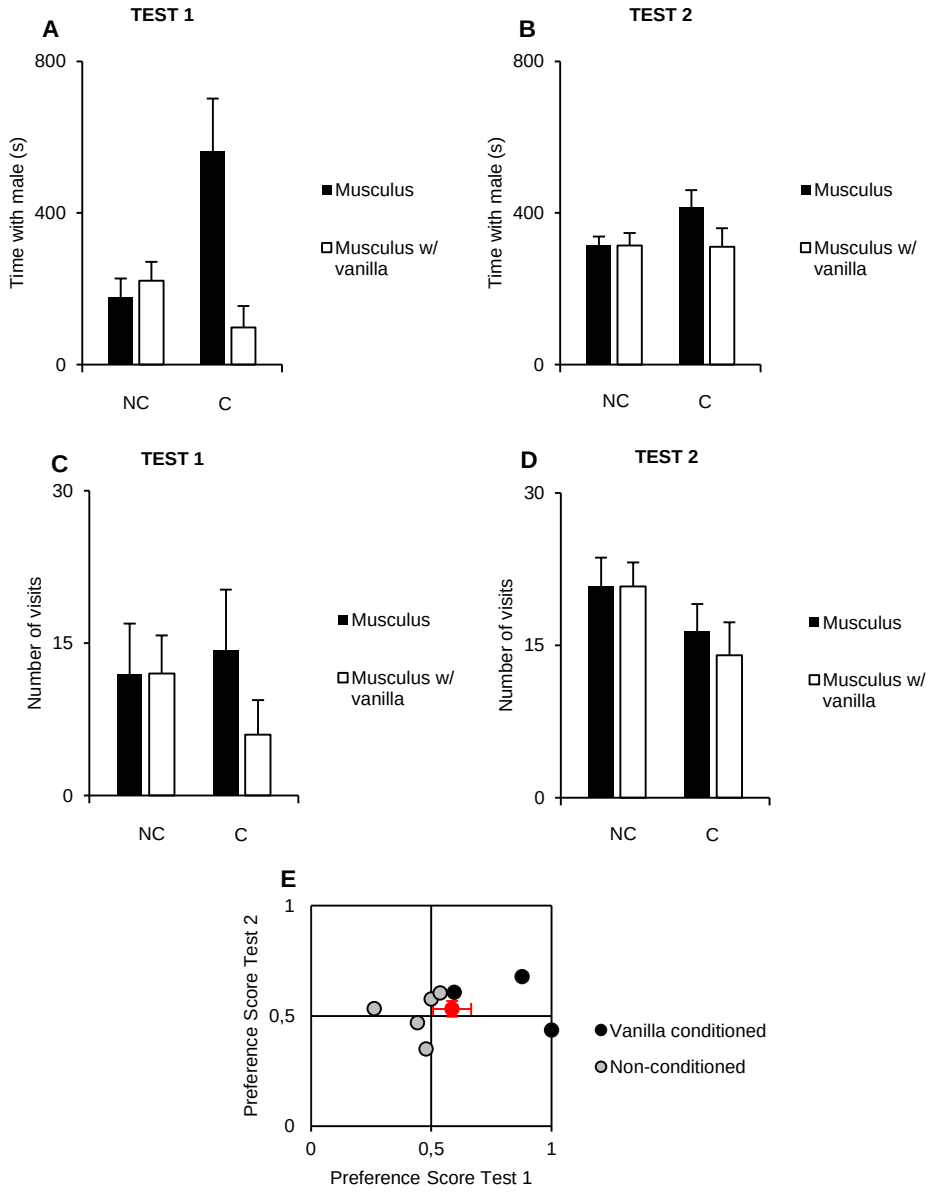

Figure 6 A) Time spent in seconds by *musculus* females with each male in the first test ("NC", Non-Conditioned: X ± SE *musculus* = 176.80 ± 55.85, N=5; X ± SE *musculus* w/ vanilla = 221.28 ± 55.63, N=5; Wilcoxon signed-rank test, V = 2, *P* = 0.273; "C", Conditioned: X ± SE *musculus* = 562.86 ± 170.30, N=3; X ± SE *musculus* w/ vanilla = 97.95 ± 69.12, N=3; Wilcoxon signed-rank test, V = 6, *P* = 0.250). B) Time spent in seconds by *musculus* females with each male in the second test ("NC", Non-Conditioned: X ± SE *musculus* = 316.32 ± 122.19, N=5; X ± SE *musculus* w/ vanilla = 313.93 ± 187.25, N=5; Wilcoxon signed-rank test, V = 9, *P* = 0.813; "C", Conditioned: X ± SE *musculus* = 415.42 ± 55.54, N=3; X ± SE *musculus* w/ vanilla = 310.51 ± 60.25, N=3; Wilcoxon signed-rank test, V = 5, *P* = 0.500). C) Visit number to each male by *musculus* female's in the first test ("NC", Non-Conditioned: X ± SE *musculus* = 12 ± 5.50, N=5; X ± SE *musculus* w/ vanilla = 12 ± 4.20, N=5; Wilcoxon signed-rank test, V = 5, *P* = 1; "C", Conditioned: X ± SE *musculus* = 14.33 ± 7.26, N=3; X ± SE *musculus* w/ vanilla = 6 ± 4.16, N=3; Wilcoxon signed-rank test, V = 6, *P* = 0.102). D) Visit number to each male by *musculus* female's in the second test ("NC", Non-Conditioned: X ± SE *musculus* = 20.8 ± 3.15, N=5; X ± SE *musculus* w/ vanilla = 20.8 ± 2.63, N=5; Wilcoxon signed-rank test, V = 8, *P* = 1; "C", Conditioned: X ± SE *musculus* = 16.33 ± 3.33, N=3; X ± SE *musculus* w/ vanilla = 14 ± 4, N=3; Wilcoxon signed-rank test, V = 5, *P* = 0.414). E) *Musculus* female's preference score for vanilla-painted *musculus* males in the first and second test (Mann-Whitney test, U = 33, N = 8, *P* = 0.959); Black and grey dots are individual data and red dot the mean ± SE

Since we found garlic to be an aversive stimulus to *musculus* females, we decided to measure both the strength of this aversion, but also how robust was the preference for *musculus* males. Therefore, we gave vanilla-conditioned females a choice between a *musculus* male scented with garlic and a *domesticus* male scented with vanilla. Conditioned and non-conditioned females showed a similar behaviour during both tests (time spent and visit number, Supplementary Fig. 3) and interestingly, a strong avoidance towards the, usually preferred, *musculus* male when painted with garlic, in the first (X ± SE P.S. for *musculus* of non-conditioned females in test 1 = 0.21 ± 0.14, N = 7; X ± SE P.S. for *musculus* of conditioned females in test 1 = 0.21 ± 0.07, N = 8; Mann-Whitney test, U = 20.5, *P* = 0.416), and second test (X ± SE P.S. for *musculus* of non-conditioned females in test 2 = 0.41 ± 0.11, N = 7; X ± SE P.S. for *musculus* of conditioned females in test 2 = 0.27 ± 0.04, N = 8; Mann-Whitney test, U = 35, *P* = 0.463) (Fig 7A).

Although we knew garlic to be an intrinsically aversive odorant for *musculus* females, we also decided to test if females who were raised by mothers painted with this odour showed a different behaviour towards garlic in adulthood. Hence, we tested conditioned and non-conditioned females when they were given a choice between a *musculus* male painted with vanilla and a *domesticus* male painted with garlic. Analysis of the female's behaviour showed that both females behaved in a similar way during both tests (time spent and visit number, Supplementary Fig. 4) and consistently avoided the garlic-scented *domesticus* male, independently of their early life olfactory experience (Fig. 7B). A summary of the results of these experiments is presented in Supplementary Table 1.

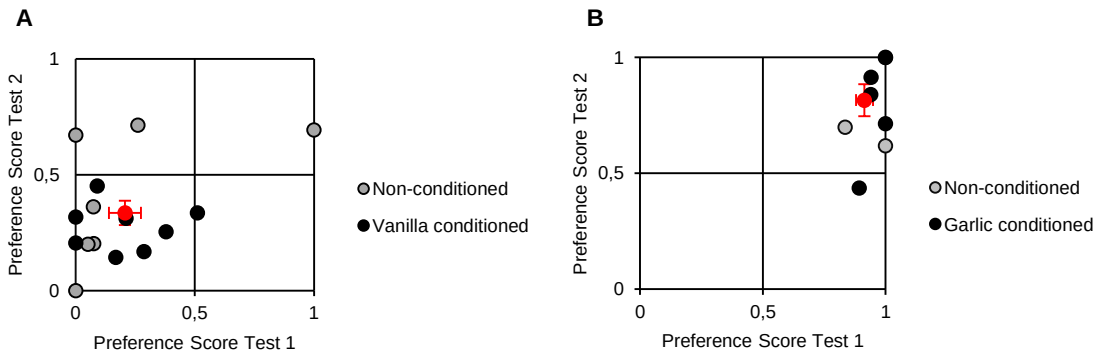

Figure 7 A) *Musculus* female's preference score for garlic-painted *musculus* males in the first and second test (Mann-Whitney test, U = 67, N = 15, *P* = 0.061). B) *Musculus* female's preference score for vanilla-painted *musculus* males in the first and second test (Mann-Whitney test, U = 38, N = 8, *P* = 0.555). Black and grey dots are individual data and red dot the mean ± SE

DISCUSSION

In this project we set out to unravel the mechanisms underlying mate choice in female mice. Using wild-derived inbred strains we confirmed the homosubspecific assortative preference of *musculus* females, which is thought to play a major role in keeping *musculus* and *domesticus* subspecies apart in the European contact zone. In the assay we use in our laboratory, this preference can be seen not only at the level of the total time spent with each male, but also trough the number of visits to both males, with a greater effect during the second test. We also confirmed that the estrous state of the females does not influence their social preferences when tested in limited contact condition, using a social preference test (SPT).

Previous results from our lab suggest that this assortative mate preference is heavily dependent on the social environment in which the females are raised, since *musculus* females fostered in a *domesticus* family lose their natural preference for *musculus* males (Zinck and Lima, unpublished). Olfaction, the main communication channel in rodents, is likely to play an important part in this process. We developed a hypothesis based on an olfactory learning process and sexual imprinting as a driving force in establishing *musculus* females mating preferences.

Several elements in the early life environment can potentially provide information and allow *musculus* females to choose a male partner in adulthood. We found the presence of the father during post-natal development to be a non-essential feature for the display of a strong assortative preference for *musculus* when compared to *domesticus* males. Females raised with and without a father showed a similar preference towards *musculus* males. These results suggest that if *musculus* females use a sexual imprinting mechanism in early life, the father is probably not a crucial element of the imprinting set. However, being this assortative preference such an important feature for the *musculus* subspecies, one can make the argument that the system through which these preferences are set could be robust enough that even if the father's presence is relevant, his removal can otherwise be compensated using other cues from the environment, like the mother or the siblings.

Alternatively, *musculus* females learning might depend on cues associated to the father, but not to his presence *per se*. Like previously mentioned, adult male mice urine convey important information about species identity (Ganem, 2001), meaning that the father's urine could be an important source of information for the females to search what to look for in adulthood. Since we wanted to reduce the stress levels of the dam and pups as much as possible, the breedings where the father was removed had its cage changed only a couple of weeks after the litter was born. This means that there was an opportunity for the newborn females

to be in contact with their father's urinary cues during part of their post-natal development.

Although less plausible, several studies have also shown that pre-natal olfactory cues can influence rodent post-natal behaviour (Smootherman, 1982), meaning that the presence of the father during most of pre-natal development might be sufficient for *musculus* females sexual imprinting. However, it is important to point out that the influence of olfactory cues in pre-natal development was always analysed by manipulating the scent of the amniotic fluid and, to our knowledge, no studies have shown the influence of external olfactory stimuli during pre-natal development, in post-natal behaviour.

To investigate the effects of early olfactory experience in the mating preferences of *musculus* females, we used artificial odours and classical conditioning to manipulate female's early life experience. We started by using citronellal, a lemon odour, to alter the scent of the home environment during the post-natal development of *musculus* females. Our prediction was that females exposed to citronellal would show in their adulthood a preference for this odour, and that it would increase the attractiveness of any male bearing this odour. However, we found no differences in the behaviour of conditioned and control females, as both groups spent significantly more time with the *musculus* male when compared with the time spent with the, citronellal-perfumed, *domesticus* male. Moreover, when the choice given was between two *musculus* males, both sets of female's still showed a strong preference for the non-perfumed male.

Since the female's preference for males of her own subspecies is a considerably robust behaviour, we hypothesized that even if the citronellal exposure had increased the value of this scent in adult conditioned females, its association to a *domesticus* male may not be sufficient to alter the value of this naturally less preferred male. Hence, we decided to test female's preference with two *musculus* males, only one of those males would be scented with citronellal. Results showed that all *musculus* females, both conditioned and non-conditioned, show a preference towards the *musculus* males without citronellal. This suggested that instead of making a male-based decision, the females were simply avoiding the citronellal, independently of which it was associated with.

To address this question, we decided to analyse the female's behaviour when they were given a choice between two pieces of paper filter, one of them being scented with citronellal. Similarly to what we had previously observed in the experiments where males were painted with citronellal, all females robustly avoided the box that contained the paper filter with the odour. These results strongly suggest that citronellal is an aversive odour for adult *musculus* females.

Several studies have used lemon odours to condition mice and none of them has ever described aversive responses in adult animals. This is the reason why we did not started our study by testing the response of both adult and newborn

females towards citronellal. Since other studies have already used pup exposure to artificial odours to study adult social behaviour, we reason that our conditioning protocol might still be useful to study *musculus* females assortative mate choice.

Since the experiments with citronellal did not allow us to test our hypothesis of an olfactory imprinting process in *musculus* females, we decided to manipulate the new-borns olfactory experience by altering the dam scent. We used vanilla and garlic odorants to paint the mothers during the entire weaning period (PN0 – PN21) and then tested the attractiveness of the females towards these odours in adulthood. Although we based our choice of odorants in a previous study where *Mus musculus* mice showed a more or less neutral response towards both these odorants (Stowers, 2012), we decided to test the response of adult *musculus* females to these scents in a non-social context. We found that adult, non-conditioned, *musculus* females showed a neutral (or slightly appetitive) response to vanilla, and a strong aversion towards garlic.

We then decided to condition *musculus* females by raising them with mothers painted with vanilla. We found this conditioning to be ineffective since conditioned females showed no increased attraction towards the vanilla-scented males. Furthermore, *musculus* females raised by garlic-scented mothers still showed a strong aversion towards males painted with this odour in adulthood. Overall, these results suggest that olfactory conditioning *musculus* females with garlic and vanilla odours does not influence adult behaviour towards these odours. However, we cannot disentangle if this happens because our protocol is insufficient to condition *musculus* females, due to the fact that the vanilla odour isn't salient enough for example, or if the conditioning did not work because exposure to non-relevant odours during early life has no impact in *musculus* females adult social preferences. Another possibility is that the conditioning procedure was efficient, but the concentration of vanilla used to test female preference was not. Further experiments, either using more salient (qualitatively and quantitatively) odours, or by finding other ways of manipulating female's post-natal olfactory experience will be needed to address this issues.

Insofar, we have seen that whenever *musculus* females are given a choice between a *musculus* and a *domesticus* male, they exhibit a strong preference for the *musculus* one. This means that, in normal circumstances, *musculus* female's value *musculus* males more than *domesticus* ones. However, this preference is not absolute. We have shown that, in both garlic conditioned and non-conditioned females, when given a choice between a garlic-scented *musculus* and a vanilla-scented *domesticus* male, their preference for *musculus* males disappears. Instead, the majority of females show a strong avoidance towards the usually-preferred *musculus* male. These results highlight, not only the female's strong aversion to garlic, but also that the assortative preference of *musculus* females is a flexible behaviour. In fact, this is in agreement with previous results in the laboratory, showing that when no-choice is allowed, and only one male is

available at a time, female's equally interact and mate with both subspecies (Lima, 2013). Hence, not only the *musculus* female's assortative preference arises from the comparison between both subspecies, but it can also be modulated by several factors, such as past experience, internal state and environmental olfactory stimuli as shown by our results. These results are in line with several recent reports that put forward the idea that female mate, rather than purely relying on, innate preferences, must be seen under the light of other cognitive processes involving learning and memory.

Overall, this study provided some insight into the role of early life experience in mate choice. We tested different olfactory conditioning protocols and evaluated the importance of the father in the development of *musculus* female's assortative preferences. The results here presented raise several interesting questions to be addressed in future experiments. For instance, the increase in preference from the first to the second test is one of the most interesting dynamics of *musculus* female's preferences. To us, this increase in the time spent with the *musculus* male might reflect a change, from the first to the second test, in the way females value males from both subspecies. Furthermore, we know that female mice need to contact the non-volatile cues in male mice urine before showing an attraction towards these cues. Darcin, an innately attractive Major Urinary Protein (MUP) in male mice urine (Hurst, 2010) could be involved in modulating these preferences. Maybe newborn *musculus* females use the father's urine to learn the olfactory profile of a *musculus* male and then use this memory in adulthood to choose an appropriate male. Interestingly, in the cross-fostering experiments previously carried out in our lab, the *musculus* females were raised with a *domesticus* strain where the males only express trace amounts of Darcin, which gives support to this hypothesis and could help explain why they did not show the typical assortative preference for *musculus* males. Addressing these questions will allows to learn more about the underlying mechanisms regulating *musculus* females mate preferences.

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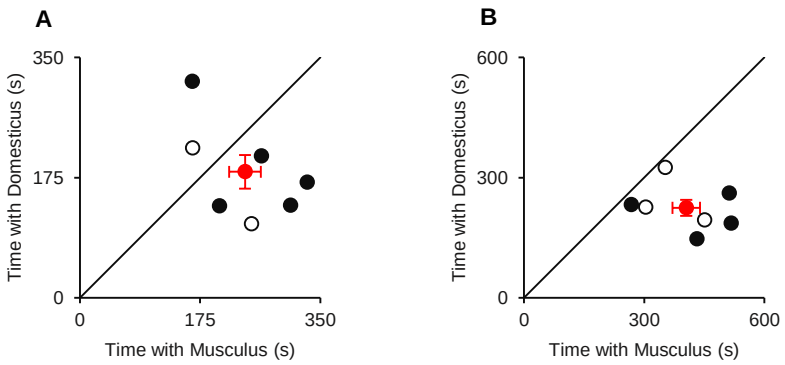
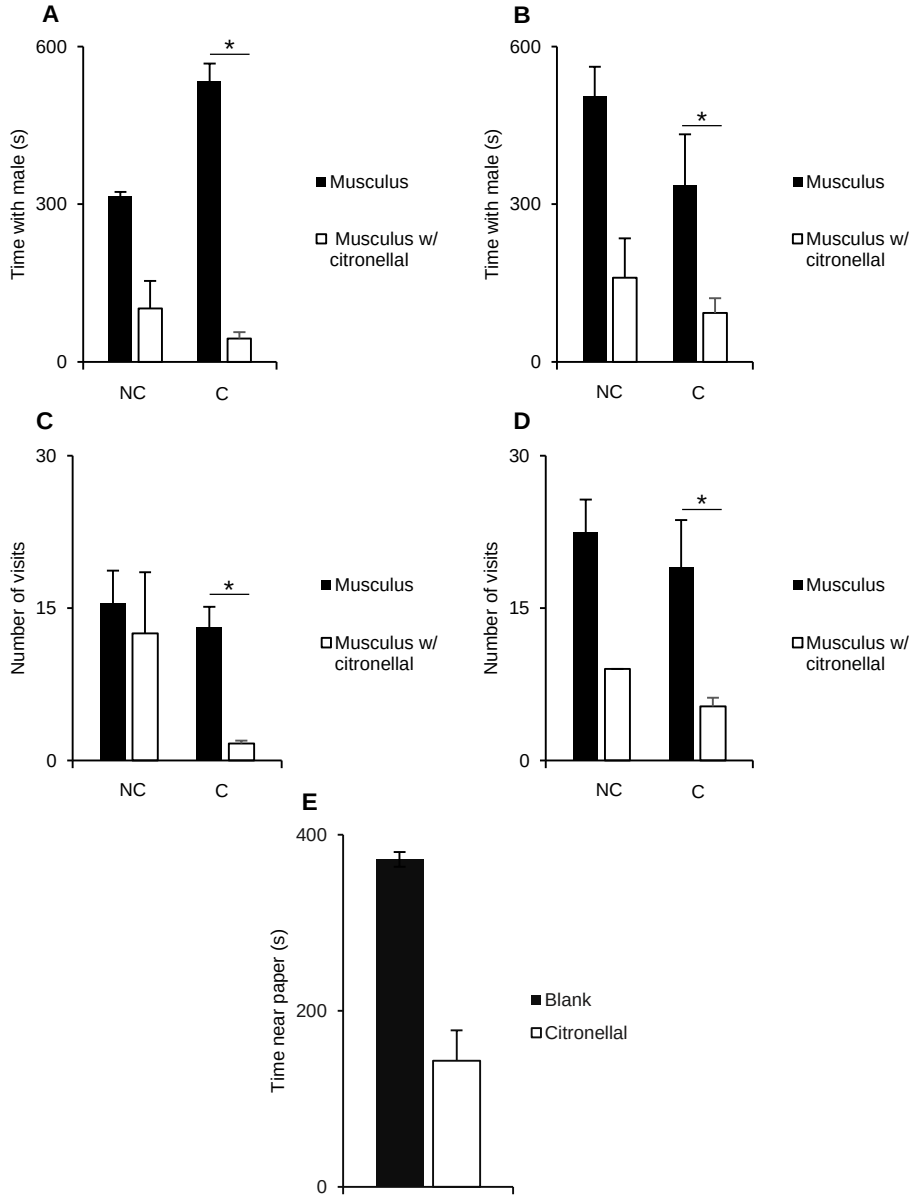
SUPPLEMENTARY DATA



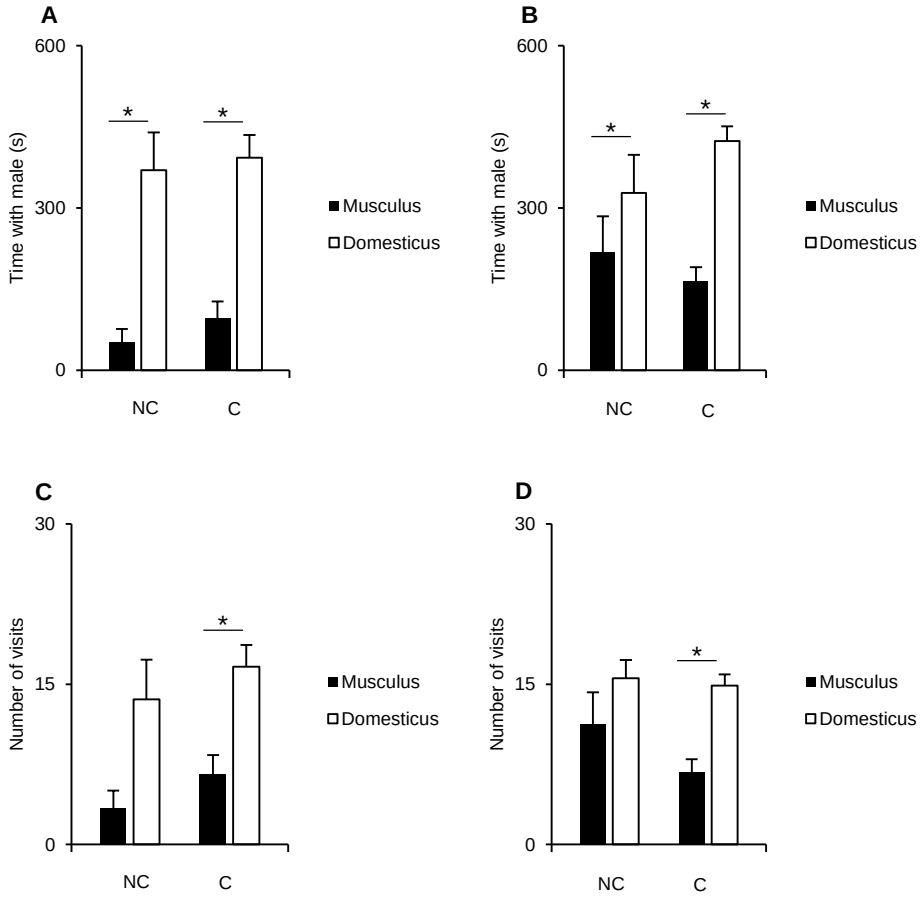
Sup. Fig. 1 A) Time spent in seconds by *musculus* females with each male in test one (X ± SE *musculus* = 240.35 ± 25.07, N=7; X ± SE *domesticus* = 183.41 ± 26.67, N=7). B) (X ± SE *musculus* = 404.76 ± 37.38, N=7; X ± SE *domesticus* = 224.58 ± 21.87, N=7); Black dots are individual data of diestrous females, white dots individual data of estrous females and red dot is the mean ± SE

SUPPLEMENTARY DATA



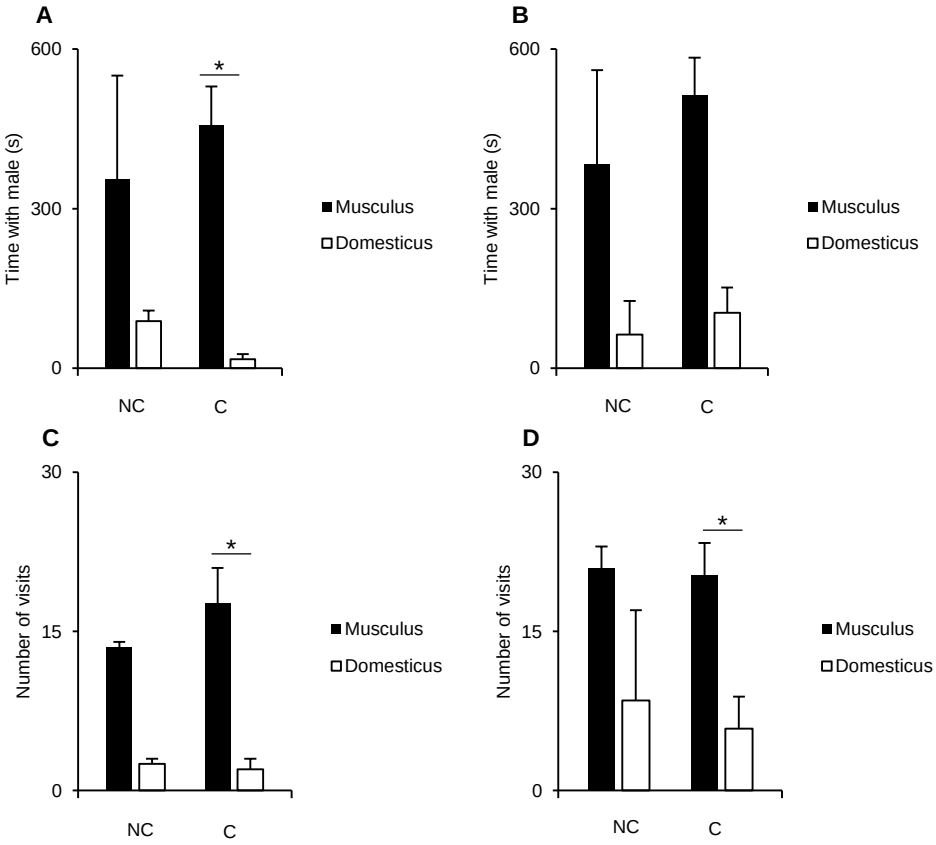
Sup. Fig. 2 A) Time spent in seconds by *musculus* females with both males in the first test (Non-Conditioned: X ± SE *musculus* = 315.07 ± 8.15, N=2; X ± SE *musculus* w/ citronellal = 101.90 ± 51.93, N=2; Conditioned: X ± SE *musculus* = 532.68 ± 35.02, N=6; X ± SE *musculus* w/ citronellal = 44.27 ± 12.16, N=6; Wilcoxon signed-rank test, V = 21, *P* = 0.031). B) Time spent in seconds by *musculus* females with both males in the second test (Non-Conditioned: X ± SE *musculus* = 505.04 ± 56.32, N=2; X ± SE *musculus* w/ citronellal = 160.21 ± 74.57, N=2; Conditioned: X ± SE *musculus* = 335.28 ± 97.71, N=6; X ± SE *musculus* w/ citronellal = 92.97 ± 28.22, N=6; Wilcoxon signed-rank test, V = 21, *P* = 0.031). C) Visit number to each male by *musculus* female's in the first test (Non-Conditioned: X ± SE *musculus* = 15.5 ± 3.12, N=2; X ± SE *musculus* w/ citronellal = 12.5 ± 6.01, N=2; Conditioned: X ± SE *musculus* = 13.17 ± 1.96, N=6; X ± SE *musculus* w/ citronellal = 1.67 ± 0.30, N=6; Wilcoxon signed-rank test, V = 21, *P* = 0.026). D) Visit number to each male by *musculus* female's in the second test (Non-Conditioned: X ± SE *musculus* = 22.5 ± 3.18, N=2; X ± SE musculus w/ citronellal = 9 ± 0, N=2; Conditioned: X ± SE *musculus* = 19 ± 4.66, N=6; X ± SE musculus w/ citronellal = 5.33 ± 0.83, N=6, Wilcoxon signed-rank test, V = 15, *P* = 0.043). E) Time spent in seconds by *musculus* females near paper filter (X ± SE blank = 372.12 ± 8.28, N=4; X ± SE citronellal = 143.31 ± 34.49, N=4; Wilcoxon signed-rank test, V = 10, P = 0.125). * means *P* < 0.05

SUPPLEMENTARY DATA



Sup. Fig. 3 A) Time spent in seconds by *musculus* females with both males in the first test (Non-Conditioned: X ± SE *musculus* w/ garlic = 52.63 ± 23.62, N=7; X ± SE *domesticus* w/ vanilla = 369.52 ± 69.72, N=7; Wilcoxon signed-rank test, V = 1, *P* = 0.031; Conditioned: X ± SE *musculus* w/ garlic = 96.91 ± 30.27, N= 8; X ± SE *domesticus* w/ vanilla = 392.85 ± 42.07, N=8; Wilcoxon signed-rank test, V = 1, *P* = 0.016). B) Time spent in seconds by *musculus* females with both males in the second test (Non-Conditioned: X ± SE *musculus* w/ garlic = 218.24 ± 66.13, N=7; X ± SE *domesticus* w/ vanilla = 327.55 ± 70.32, N=7; Wilcoxon signed-rank test, V = 9, *P* = 0.05; Conditioned: X ± SE *musculus* w/ garlic = 164.84 ± 27.80, N= 8; X ± SE *domesticus* w/ vanilla = 423.29 ± 27.67, N=8; Wilcoxon signed-rank test, V = 0, *P* = 0.05). C) Visit number to each male by *musculus* female's in the first test (Non-Conditioned: X ± SE *musculus* w/ garlic = 3.43 ± 1.62, N=7; X ± SE *domesticus* w/ vanilla = 13.58 ± 3.73, N=7; Wilcoxon signed-rank test, V = 3, *P* = 0.078; Conditioned: X ± SE *musculus* w/ garlic = 6.63 ± 1.73, N= 8; X ± SE *domesticus* w/ vanilla = 16.63 ± 2.04, N=8; Wilcoxon signed-rank test, V = 0, *P* = 0.012). D) Visit number to each male by *musculus* female's in the second test (Non-Conditioned: X ± SE *musculus* w/ garlic = 11.29 ± 2.96, N=7; X ± SE *domesticus* w/ vanilla = 15.57 ± 1.70, N=7; Wilcoxon signed-rank test, V = 7.5, *P* =0.271; Conditioned: X ± SE *musculus* w/ garlic = 6.75 ± 1.24, N= 8; X ± SE *domesticus* w/ vanilla = 14.88 ± 1.04, N=8; Wilcoxon signed-rank test, V = 0, *P* = 0.018). * means *P* < 0.05

SUPPLEMENTARY DATA



Sup. Fig. 4 A) Time spent in seconds by *musculus* females with both males in the first test (Non-Conditioned: X ± SE *musculus* w/ vanilla = 355.57 ± 194.41, N=2; X ± SE *domesticus* w/ garlic = 88.70 ± 19.47, N=2; Conditioned: X ± SE *musculus* w/ vanilla = 457.09 ± 72.53, N= 6; X ± SE *domesticus* w/ garlic = 16.69 ± 9.66, N=6; Wilcoxon signed-rank test, V = 21, *P* = 0.031). B) Time spent in seconds by *musculus* females with both males in the second test (Non-Conditioned: X ± SE *musculus* w/ vanilla = 382.70 ± 177.72, N=2; X ± SE *domesticus* w/ garlic = 63.05 ± 63.04, N=2; Conditioned: X ± SE *musculus* w/ vanilla = 513.64 ± 70.14, N= 6; X ± SE *domesticus* w/ garlic = 104.38 ± 47.58, N=6; Wilcoxon signed-rank test, V = 20, *P* = 0.063). C) Visit number to each male by *musculus* female's in the first test (Non-Conditioned: X ± SE *musculus* w/ vanilla = 13.5 ± 0.5, N=2; X ± SE *domesticus* w/ garlic = 2.5 ± 0.5, N=2; Conditioned: X ± SE *musculus* w/ vanilla = 17.67 ± 3.32, N= 6; X ± SE *domesticus* w/ garlic = 2 ± 1, N=6; Wilcoxon signed-rank test, V = 21, *P* = 0.031). D) Visit number to each male by *musculus* female's in the first test (Non-Conditioned: X ± SE *musculus* w/ vanilla = 21 ± 2, N=2; X ± SE *domesticus* w/ garlic = 8.5 ± 8.5, N=2; Conditioned: X ± SE *musculus* w/ vanilla = 20.33 ± 3, N= 6; X ± SE *domesticus* w/ garlic = 5.83 ± 3.03, N=6; Wilcoxon signed-rank test, V = 15, *P* = 0.043). * means *P* < 0.05

SUPPLEMENTARY DATA

Dam scent manipulation	Test	Preference
None	garlic vs. water	water
None	vanilla vs. water	none
Vanilla	Mus ♂ + vanilla vs. Mus ♂ + water	none
Vanilla	Mus ♂ + garlic vs. Dom ♂ + vanilla	Dom ♂ + vanilla
Garlic	Mus ♂ + vanilla vs. Dom ♂ + garlic	Mus ♂ + vanilla

Sup. Table 1. Summary of results from “Early experience manipulation 3: Manipulation of dam scent does not impact the social preference of musculus females”.