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Dissertação

Mestrado em Biologia Evolutiva e do Desenvolvimento

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Orientador: Doutora Susana Lima

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Abstract

Sexually reproducing species guarantee the continuity of the species by means of sexual interactions. Animals make use of innate and learned actions to increase the probability of being in contact with an animal of the opposite gender leading to *copulatory/consummatory* behaviour. After ejaculation is reached, sexual interest is diminished and animals enter a *refractory state.* Mice sexual behaviour is characterised by a series of mount attempts and mounts-with-intromissions, which in the end eventually lead to ejaculation, with this study we aimed to understand if there were male sexual behaviour between to major subspecies of mices, *Mus musculus musculus* and *Mus musculus domesticus*. We showed that there are differences in male sexual behaviour, but unfortunately we could not determine if those differences arise from the fact that this different strains fit in different subspecies of mice. *M. musculus* females showed to drastically change *M. domesticus* behaviour making those males necessity less genital stimulation to reach ejaculation (quality versus quantity) and finally with the employed method we could not give more insight about the Prolactin role in establishing or maintaining PERP.

Keywords: *Mus musculus musculus: Mus musculus domesticus*; Sexual behaviour; Post-ejaculatory refractory period; Prolactin

Resumo

De modo a garantir a continuidade de espécies que se reproduzem sexualmente, é necessário que indivíduos portadores dos dois tipos de gâmetas interajam, de modo que a sua informação genética seja transmitida à prole. Contrariamente a outros comportamentos, como alimentação ou sono, que são fundamentais para a sobrevivência do indivíduo, o comportamento sexual não é fundamental mas a natureza evoluiu mecanismos que garantem a execução de comportamentos, ditos sexuais, que levam à junção dos dois gâmetas. Sendo assim, o comportamento sexual pode ser visto como um ciclo que compreende três fases distintas: uma fase que depende de sinais internos e externos que levam a que o indivíduo aumente a execução de acções, fase apetitiva; a fase apetitiva aumenta a probabilidade do par executar a cópula, fase comportamento consumatória; durante a cópula, haverá a junção dos dois gâmetas e geralmente este passo está correlacionado com uma diminuição do interesse sexual, chamada fase inibitória.

Todas estas fases do comportamento sexual são controladas por zonas distintas do cérebro e dependem de diferentes sistemas de neurotransmissores e hormonas para a sua execução. Embora muitos dos estudo existentes tenham sido realizados em humanos, grande parte do nosso conhecimento corrente teve origem em estudos realizados em animais, principalmente o rato, Mus musculus. No entanto, a espécie Mus musculus pode ser ainda dividida em três subespécies diferentes: Mus musculus musculus, Mus musculus domesticus e Mus musculus castaneus que vivem em diferentes habitats e que apresentam diferentes comportamentos, nomeadamente, na escolha do seu parceiro sexual. Por exemplo, estudo realizados por vários grupos e pelo nosso laboratório, mostram que enquanto que a fêmeas musculus escolhem os seus parceiros e preferem acasalar com membros da sua própria subespécie quando lhe é dada a escolha entre um macho musculus e um macho domesticus, as fêmeas domesticus acasalam indiscriminadamente com machos das duas subespécies. Este facto levou-nos a supor que, além das diferenças em termos de escolha de parceiro, poderão haver outras diferenças em termos do comportamento sexual. Para isso, o objectivo principal desta tese de mestrado foi comparar o comportamento sexual do macho das duas subespécies de rato *Mus musculus musculus* e *Mus musculus domesticus*. Este tipo de análise comparativa tem sido bastante usada e tem levado a resultados muito importantes. Isto porque permite estudar espécies com um ancestral comum mas que devido à sua história evolutiva, divergiram em termos de comportamento. Por isso, permitem-nos investigar as bases neuronais para as diferenças apresentadas, uma vez que o cérebro destas espécies é muito semelhante. Além disso, queríamos investigar possíveis mecanismos hormonais/neuronais responsáveis por estas diferenças entre subespécies.

Neste projeto centrámo-nos no comportamento consumatório e inibitório do macho, uma vez que é relativamente fácil identificar estas duas fases do comportamento sexual: o comportamento consumatório é caracterizado por uma série de acções em que o macho agarra a fêmea e a monta, levando à introdução do pénis na vagina; esta fase é também caracterizada por uma série de montas e desmontas, em que os dois animais interagem e depois se separam; a ejaculação também é facilmente identificada, uma vez que o macho agarra a fêmea e colapsa, mantendo-se imóvel durante uns segundo. Em termos da fase inibitória, é também fácil isolar este comportamento pois é caracterizada por uma falta de interesse sexual na fêmea. Esta fase termina quando o macho volta a ganhar interesse sexual e novamente monta a fêmea. Sendo assim, a primeira experiência deste projecto foi comparar a performance sexual dos machos das quatro estirpes mencionadas; a análise foi feita em vários parâmetros comportamentos que permitem identificar as fases consumatórias e inibitória.

O nosso objectivo nesta primeira experiência era perceber se os animais de cada subespécie poderiam realmente ser agrupados e podermos estabelecer um comportamento dito próprio da subespécie musculus, diferente da espécie domesticus. Embora não tenha sido possível diferenciar em subespécies todos os comportamentos analisados, os nossos dados indicam que as duas strains de machos musculus necessitam menos estimulação genital para atingir a ejaculação. Não sabemos se este fenómeno está relacionado com a ecologia comportamental desta espécie ou com o facto das estirpes escolhidas para este estudo (PWD e PWK) terem uma origem mais recente e terem passado menos tempo em laboratório. Experiências futuras serão necessárias para tomar uma conclusão.

Uma vez que a performance sexual do macho não depende apenas da motivação e comportamento do macho, a decidimos investigar qual o peso do comportamento da fêmea para os resultados obtidos anteriormente. Para isso realizámos experiências em que os machos de cada strain podiam interagir com fêmeas das outras strains. Deste modo poderíamos estudar como é que o comportamento de cada macho se altera quando tem que copular com fêmeas diferentes. Aqui, surpreendentemente, os resultados mostraram que enquanto que os machos da subespécie musculus não parecem mudar de um modo significativo o seu comportamento quando interagem com fêmeas musculus ou domesticus, os machos domesticus mudam. Os machos domesticus passam a precisar de menos estimulação genital para atingir a ejaculação. Isto parece sugerir que as fêmeas musculus levam a uma cópula mais recompensador ou a uma maior excitação que faz com que menos estimulação sensorial seja necessária. Mais uma vez, não sabemos se o efeito das fêmeas musculus tem origem em serem uma outra subespécie ou o facto de serem mais selvagens do que os machos domesticus.

Finalmente, tentámos começar a investigar uma hormona que pudesse explicar as diferenças observadas em termos de comportamento sexual. Além dos resultados mencionados em termos de comportamento sexual e necessidade de estimulação genital, a recuperação de interesse sexual foi outra medida que permitiu diferenciar as duas subespécies: enquanto que os machos musculus recuperaram a atividade sexual em menos de duas horas após a ejaculação, quase nenhum dos machos domesticus o fez. Mais uma vez, não sabemos se este resultado provém de uma diferença real das duas subespécies ou de um artefacto derivado de um diferente historial. No entanto, independentemente da origem, esta diferença comportamental poderá ser importante para estudos futuros cujo objectivo seja perceber os mecanismos neuronais subjacentes ao controlo do período inibitório. Decidimos então olhar para a activação no cérebro causada pela hormona prolactina, uma hormona que está envolvida na lactação, mas que nos últimos anos também foi implicada em comportamento sexual. Nomeadamente, sabe-se que há um pico de prolactina após a ejaculação e pensa-se que esta libertação leve ao início do período refratário em que o macho não tem interesse sexual. Uma vez que as duas subespécies exibiram comportamento inibitório tão distinto, perguntámos se o cérebro destes animais poderá responder à prolactina de modo distinto. Para isso injéctamos machos das quatro strains com prolactina e investigamos zonas do cérebro que possam responder a esta prolactina. Olhámos para três zonas que estão envolvidas em comportamento social-sexual no hipotálamo: arcuate, núcleo paraventricular e zona pré-óptica. Não observámos diferença nenhuma em termos de activação pelo que podemos concluir, que com o método usado, a prolactina não pareça ser responsável pelas diferenças observadas.

Palavras- chave: *M. musculus*, *M. domesticus*, preferência sexual, período refractário, Prolactina.

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Introduction

Sexual behaviour

To ensure species continuity it is necessary to transfer genetic material to future generations. Sexually reproducing species guarantee this by means of sexual interactions between individuals carrying two different types of gametes, making sure each one them passes part of its genetic information to the offspring. Despite being a purposive behaviour, such as feeding [1], when considering it in an individual perspective, animals' survival does not depend on sexual interactions. Still nature developed ways to ensure that animals engage in sexual behaviour so to disperse their genetic information [2 3]. Sexual behaviour, like other motivated behaviours [3] depends on internal and external signals to ensure the appropriate execution of actions that lead to the fertilisation of the oocyte. Animals make use of innate and learned actions to increase the probability of being in contact with an animal of the opposite gender leading to *copulatory/consummatory* behaviour. At some point, which is usually correlated with the transfer of gametes, sexual interest is diminished and animals enter a *refractory state* [4 5]. Therefore, and like other motivated behaviours, sexual behaviour can be seen as a cycle that is composed of three main different stages: *wanting/appetitive* phase, *liking/consummatory* phase and an *inhibitory/satiety* phase [3].

For mammals to engage in sexual interactions, both male and female have to recognise the presence of an individual of the opposite gender and its willingness to engage in sexual behaviour. *Appetitive* stage is triggered by unconditional sexual stimuli such as pheromones, vocalisations, olfactory cues present in urine and other corporal secretions [6 7]. All these cues allow the individual to recognise the internal state of the other individual. In rodents, this behavioural stage is characterised by actions such as anogenital and nose-to-nose investigations and solicitation, which are courtship behaviours that promote sexual interaction [2 3 4 8 9 10 11 12].

In sexually naïve males, the transition from *appetitive* to *consummatory* behaviours (*consummatory stage*) is thought to be caused by accidental tactile stimulation of the pelvic reflex that leads to pelvic thrusting [2]. By performing behaviours that lead to tactile stimulation, animals learn to associate appetitive behaviours with the occurrence of tactile stimulation and importantly, with, genital stimulation [2]. Both tactile and genital stimulation are innately rewarding events, like food and water consumption [2 13]. To males, genital stimulation is intrinsically connected with the penis; the penis can be seen as a specialised organ for receiving somatosensory stimuli, highly innervated and connected to the brain [3]. Morphological studies have shown that the receptors present in the penises glans are similar across species and different from other types of receptors along the skin [3]. Sensory stimulation received by the penis leads to the induction of the penile erection that relies on the communication between

neural and humoral mechanisms at different levels of the neuroaxis [14]. In females there is not a distinct structure homologous to the penis and, both the vulva and the clitoris seem to contribute to establish the link between tactile stimulation (genital stimulation) and pleasure [15] [16]. It is though that the sensory stimulation received during sexual interaction reaches a threshold which then leads to activation of inhibitory mechanisms, ensuring the arrest of sexual desire and the beginning of *inhibitory/satiety* stage [4].

The delimitation between *consummatory* and *inhibitory* stages of sexual behaviour is perceived, in males, by the occurrence of ejaculation, however in females there's not a specific behavioural output that discriminates these two stages. Ejaculation can be part of the orgasm experience in humans, where men experience involuntary muscular contractions, sharp peaks in cardiovascular arousal, and subjective feeling of release [3] [17]. Similar physiological changes are observed in most mammals [3]. Upon experiencing ejaculation, males enter in a period termed as post-ejaculatory refractory period (PERP) in which they don't engage in sexual interactions for a certain period of time.

Brain, neuropeptides and hormones in sexual behaviour

Throughout the different stages of the sexual cycle, several brain regions are differentially engaged, as it was shown with immediate early genes (IEG) [3] [18] [19] [20]. During the *appetitive phase* it is observed the activation of hypothalamic areas such as the Medial Preoptic Area (MPOA), an area related with the processing of sensory information transmitted by the other individual [21]. The perception of the sensory information ultimately promotes the beginning of the *consummatory stage* and the sexual intercourse *per se*. During consummatory behaviours and ejaculation other hypothalamic nuclei are negaged, as well as the basal forebrain, the thalamus, the amygdala and the hippocampus [19] [21] [22] .

The activation of specific brain regions is related with an increase and decrease on the release of neuropeptides in the nervous system and hormones in the circulatory system [23]. Neuropeptides are extremely relevant for the shaping of sexual function within the central nervous system [14] [23] and, different neuropeptides and hormones exert a specific role in particular aspects of sexual behaviour. The most referenced neuropeptides and hormones involved as neuromodulators of sexual behaviour are the opioid peptides, dopamine, norepinephrine, serotonin, endocannabinoids, oxytocin and more recently prolactin [3] [4] [14] [23] [24] [25] [26].

The Dopaminergic system is considered to be part of the complex network responsible for the sexual excitement. When a male is presented with a receptive female, Dopamine (DA) levels increase in anticipation and during copulatory actions [4]. Diverse studies show that lesions in different brain areas and the injection of DA agonists in

the brain induce changes in the behavioural output. Lesions in Nucleus Accumbens (NAcc) disrupt the ability to perceive sexual cues that elicits sexual arousal; lesions in MPOA seem to disrupt normal appetitive behaviours [4] [27]. On other hand, the injection of DA agonists in the Paraventricular Hypothalamic Nucleus (PVN) induces the penile erection by activating the oxytocin (OXT) expression [14]. OXT is responsible for the induction of penile erection and also facilitates copulatory behaviours, as seen by the injection of oxytocin agonists. An increase of central oxytocin by injection leads to a shorter refractory period in male rats as the induction of penile erections [23].

Opioid peptides have shown to be related to different behaviour phenotypes depending on the brain area in which they are being expressed. For instance the injection of an opioid agonist in MPOA inhibits the *consummatory* phase of sexual behaviour, injection in the PVN seems to impair the penile erection, and injection in the Ventral Tegmental Area (VTA) accelerates the anticipatory phase by increasing the activation of dopamine [23].

Feeding studies showed that serotonin was related with the *satiety phase* [28]. In sexual behaviour, injections of serotonin caused a delay in the ejaculation after a male already started mounting the female and, on the other hand, the blockage of serotonin release in males showed to decrease the refractory period [24]. High levels of central serotonin are correlated with an inhibition of release of DA, leading to the thought that serotonin is a reasonable inhibitor of appetitive behaviours, by leading the behaviour to the sexual satiety stages [4].

Endocannabinoids have also an inhibitory role in sexual behaviour; the administration of morphine, heroin and methadone (endocannabinoid type of drugs) in rats showed to decrease the copulatory behaviour of males and females [4] [29]. On the contrary, the injection of an opioid antagonist (naloxone and naltrexone) showed to facilitate the engagement of copulatory actions and to anticipate ejaculation [30]. Endocannabinoids then seem to be related to the state of sedation in which there is less response to the stimuli, making somatosensory awareness almost inexistent [4].

Another key player that has been recognised in the regulation of sexual behaviour is prolactin (PRL). Prolactin was first described for its function on the milk production [31] but it has a role in more than 300 biological functions. PRL is a polypeptide hormone that is mainly produced and secreted by the Lactotrophs, cells found in the anterior pituitary gland, and responsible for the release of PRL in the circulatory system. Human studies showed that during an orgasm/ejaculation, self-induced or by sexual intercourse, men and women experience a peak in serum PRL [26] [32] [33] that seems to hold for a 60 minutes period [33]. Physical stimulation *per se* is not responsible for this

peak since this phenomenon is only observed when ejaculation/orgasm is reached [17] [26] [34]. The same phenomenon has been observed in other mammals [35]; [36] [37].

By recognizing the importance of DA in sexual arousal and the negative feedback loop that it establishes with PRL [1] it is hypothesised that PRL may be a key player in post-ejaculatory refractory period [34]. But, despite the several lines of evidence supporting the idea that PRL is involved in PERP and in the diminishing of sexual interest, there is still controversy in this field [38] [39].

Post-ejaculatory refractory period

PERP comprises the time period when the males are not aroused by the presence of a receptive female [39]. PERP can be classified in two different stages, **absolute refractory period** and **relative refractory period** [39]. Absolute refractory period corresponds to the period where the male is unresponsive even in the presence of an accessible receptive female. Oppositely, in the relative refractory period, a string positive stimuli (such as an accessible, new, female) can elicit arousal in the male [39].

The presence of PERP in the inhibitory stage of the sexual behaviour cycle across different mammalian species is thought to be a mechanism that prevents the males of being riskily exposed to excessive predation and parasites [4] [40] [41].
Even though PERP is a highly conserved phenomenon in mammalian sexual behaviour, its duration is highly variable. If we take a closer look to the sexual behaviour of rats and hamsters, it is noticeable that after ejaculation males only take a few minutes (10 to 12 minutes) to recover from PERP and re-gain the capacity to engage again in sexual interactions) [42] [43]. Contrarily, in mice and other species, PERP duration can vary between a few hours to more than 24 hours [44]. The high variability in the recovery time from PERP between different species can be thought as a consequence of the species ecology, physiology and selective pressures that they are exposed to. Currently, it remains ambiguous which selective pressures shaped the duration of PERP, but having in mind that we are talking about a phenomenon that is intrinsically connected with sexual behaviour, it is natural to think that sexual selection mechanisms are underlying PERP recovery time.

Originally, sperm competition was defined as a sexual selection mechanism present in insects, however it is known that sperm competition is also acting in mammals. Although mammalian females do not have the capacity to store sperm, in a wide range of species, females tend to be promiscuous and copulate with more than one male during the same oestrous cycle, enabling sperm from different males to compete for the access to the available oocytes [45]. Despite the fact that sperm competition enters in what is considered *postcopulatory selection mechanisms*, its presence favours males

that adopt physiological, morphological and behavioural strategies to incapacitate and/or displace the sperm of other males [46]. Strategies such as the development of enlarged glans penises and the deposition of copulatory plugs in the female reproductive tract are examples of strategies that seem evolved under a sperm competition context [41 46 47].

Behavioural features like multiple ejaculations, multiple intromissions and PERP recovery time are among the diverse behavioural features that show to be shaped by sperm competition environments. Studies conducted mainly in rodent species [41 46 47 48] have shown that the increase of sperm competition is strongly correlated with more frequent ejaculations, more intromissions per mount and shorter PERP recovery time [46]. Multiple ejaculation strategy increases the probability of a male to sire more offspring within each sexual encounter, since males allocate only a portion of all the available spermatozoids at each ejaculation. This maximizes sperm transfer under conditions where there is the risk of interruption by other male [41 46 47]. Sperm allocation strategies are normally reported in species and environments where males are under a high level of competition regarding the access to females [41 46 48 49]. Such evidences support the idea that PERP recovery time is correlated with sperm competition environments [46]. By being under different sperm competition pressures, populations/species obtained different optimal copulatory strategies to optimise the probability to sire more offspring, and therefore changing different copulatory behaviours, such as the number of duration of animal contact [41 46].

Mice sexual behaviour

Copulatory behaviour in small rodents has been studied in more detail in the last decades. Sexual behaviour in mice follows a recurring cycle with different phases, as in the case for other mammals. Male mice mating patterns are characterised by a series of mount attempts and mounts-with-intromissions, which in the end eventually lead to ejaculation [8 9 50]. The male grabs the female with his forepaws and executes pelvic thrusts. During the mount period, the female plants her feet, raises her hindquarters and her tail (*lordosis posture*) [8]. When ejaculation is reached, male mice normally fall to their side still mounting the female for a variable period of time [8]. As in the case of other rodents, mice also show the capability to recover from PERP in a short period of time and re-engage in sexual behaviour [8 41 46]. Interestingly, different lab strains of mice showed a different performance when accessed for the recovery time of PERP, ranging from one hour to four days [8].

Mice have been one of the most useful species in laboratory research in fields such as genetics [51]. The establishment of hundreds of laboratory inbred strains have helped to pursue the answer for different biological questions. Different strains are used in different fields of research exhibiting genotypic, physiological and behavioural

differences. Most mice used in research belong to the Mus musculus species (house mouse) [52]. However, this species can be further divided into three different subspecies: Mus musculus musculus, Mus musculus domesticus and Mus musculus castaneus [53]. A lot of effort has been devoted to study the genetic mechanisms underlying the differences between musculus and domesticus animals in particular because they maintain their identity even though they can hybridize along their contact zone [54]. Several studies have shown that females from musculus and domesticus animals exhibit different preference behaviour: while musculus females are picky and prefer males from their own subspecies, domesticus females mate indiscriminately with males from both [54]. This indicates that both species have different strategies in terms of sexual selection and might also have different characteristics in terms of sexual behaviour.

Goals

Having as a starting point the fact that the sexual preferences of musculus and domesticus animals is different, the aim of this project was to investigate the sexual behavioural strategies of these two subspecies of mice.

Therefore the main goals were:

- Compare the sexual behaviour of male *Mus musculus musculus* (PWK/PhJ and PWD/PhJ) and *Mus musculus domesticus* (C57BL/6J and Balb/cJ)
- Investigate how male sexual behaviour is affected by females of different subspecies
- Ultimately, having in consideration the human studies (Kruger et al. 1998; Exton et al. 2000) that report a possible role of PRL in PERP, determine and compare brain regions that can be modulated by PRL in both subspecies.

Materials & Methods

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 [52 55]. As representative of the *Mus musculus domesticus* subspecies we used the classical laboratory strains C57BL/6J and Balb/cJ also ordered from The Jackson Laboratory. This and other combination of strains have been previously used to study the behavioural ecology and genetics of these two subspecies of house mouse [52 54 55] . All animals were weaned at 21 days of age and housed in same-sex groups of two to five 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 in the afternoon at least 4 hours after light onset. Cage changing was performed once per week for *domesticus* animals and once every other week for *musculus*. To stimulate proper olfactory development, all females were exposed to male soiled bedding and 10 µL of male urine in alternating weeks. Both the soiled bedding and the urine were a mixture of equal volumes from PWK/PhJ, PWD/PhJ and C57BL/6J males. Males used for experiments where between 75 days and 180 days of age and were isolated to smaller cages prior to experiments (1145T, Techniplast, 369 x 156 x 132 mm). Animals were sacrificed by CO2 asphyxiation followed by cervical displacement at the end of each set of experiments.

Sexual behaviour characterisation experiments

In order to decrease the variability in female behaviour and ensure sexual receptivity, all females were ovariectomized and treated with sexual hormones to induce a behavioural estrous. In summary, females underwent a bilateral ovariectomy (OVX) (surgeries were conducted under isofluorane anaesthesia) and were after primed with Estradiol Benzoate and Progesterone before the experiments. Females were injected with 5 µl of Estradiol Benzoate in 0.1 ml of Sesame oil, followed by 500 µl of progesterone in 0.1 ml sesame oil 4h before testing. All behavioural tests were done in the male home cages (1145T, Techniplast, 369 x 156 x 132 mm).

During the first session, the *training session*, *naïve* males interacted with receptive and previously trained OVX females during a maximum of two hours. In that period the males were expected to engage in sexual behaviour and reach ejaculation. After reaching ejaculation the trial finished and the female was removed from the male home cage. The *training session* had the intention to train the male in sexual behaviour

since after weaning age they were separated from the females and did not receive any female cues.

Four days later, those that ejaculated were moved to a second session, the *refractory period session*. On that session, males had a maximum of 90 minutes to ejaculate. Subsequently, the males had up to 120 minutes to recover sexual interest with the same female. Recovery from the refractory period was identified as the first mount with intromissions after ejaculation. Each trial finished when either the first mount with intromission after ejaculation occurred or by the end of the 120 minutes.

Males that did not engaged in sexual behaviour or that did not reached ejaculation in the time established, repeated the session until fulfil the experimental conditions. All the sessions were video recorded, and the analysis was conducted in the *refractory period session* of each individual.

Each male was assigned to a specific experimental condition being exposed in all sessions to different females, but always from the same strain (Table.1)

♂ \ ♀	**BL6**	**PWD**	**PWK**	**Balbc**
BL6	N=19	N=16		
PWD	N=18	N=17	N=17	
PWK	N=9	N=8	N=10	
Balbc				N=8

Table.1 Number (N) of males used in each interaction:

STAT5 Immunohistochemistry protocol

Six males of each strain (PWK, PWD, BL6, Balbc), with the exception of Balbc (four males) received one of two treatments (3+3, 2+2): ovine prolactin (5 mg/kg injection/intraperitoneal (ip) 45 minutes prior to perfusion, n=5) or vehicle (saline, ip, 45 minutes prior to perfusion, n=5) injections. Animals were anesthetized with ketamine + medetomidine (0,1 ml per 10 g), the mice were then perfused transcardially with 4% paraformaldehyde. Brains were removed, postfixed in the same fixative overnight, and cryoprotected in 30% sucrose. Three sets of 30- µm coronal sections were cut on cryostat and placed in superfrost slides. From the three sets only one was used to label the activation of the phosphorylated STAT5 (pSTAT5).

The slides were washed in TBS (Tris-buffered saline 0.05M, pH 7.6) before proceeding to antigen retrieval step. Slides were incubated for 5 minutes in 0.01MTris-HCL (pH 10) at 90°C, and then cooled at room temperature for a further 40 minutes in the same solution. To block the nonspecific binding of the primary antibody, the slides were

incubated for 10 minutes in an incubation solution (2% normal goat serum, 0.3% Triton X-100 and 0,25% BSA in 0.05M TBS, pH 7.6) followed by a washing step in TBS.

Blockage of endogenous peroxidades (0.1% H202 30% / 40% methanol / TBS) was done before slides were incubated in pSTAT5 primary antibody (pSTAT5 Tyr 694, 1:400; Cell Signaling Technology, Beverly, MA) for 72 hours at 4°C. Primary antibody incubation was followed by a series of TBS washing and by biotinylated goat antirabbit IgGs (1:200 for 90 minutes; Vector Labs, Peterborough, UK). Washing step in TBS precede the Vector Elite avidin-biotin-horseradish peroxidase complex incubation (1:100 for 90 minutes). Peroxidase labeling was visualized with nickel-diaminobenzidine tetrahydrochloride using Hydrogen peroxidase (adaptation from: Brown et al. 2010).

Behaviour 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 *training* and *refractory period trials* for each male were recorded. Analysis of behaviour was performed using BONSAI software [56]. A set of behaviours were annotated during the analysis of all *refractory period sessions'* videos (**Ethogram**). From the annotated behaviours we defined six measures that gave quantitative aspects of the sexual behaviour (**Quantitative measurements of sexual behaviour)**.

a) Ethogram:

Female in (FI) – Beginning of behavioural trial. Female is placed in the male home cage.

Mount with intromission (Start/End of mount) – Male grabs the female's side and executes a series of pelvic thrusts intromitting the penis in the female vagina for a variable period of time.

Mount Attempt (M. Att) – Males tries to gain access to the female but due to inexperience or unreceptivity of the female he is not capable to engage in a series of mount with intromission. Male does unsuccessfully pelvic thrust or female tries to run away from the male.

Ejaculation (E) – Male is actively mounting the female when, in most of the cases, the male falls to the side carrying the female over with him, if not, it is visible that he remains in the mounting position tightly grasping the female and in both cases maintaining genital contact. After a certain period of time, male and females go separate ways and start to groom.

b) Quantitative measurements of sexual behaviour:

Number of mounts (N^0 mounts) – Number of mounts preceding ejaculation

Number of mount attempts (N^0 M. Att) – Number of mount attempts preceding ejaculation

Average Mount Duration (MD) – The length of mounts with intromissions before ejaculation

Inter mount interval (IMI) – Elapsed time between two consecutive mounts with intromission

Ejaculation latency (EL) – Elapsed time between the first mount with intromission and the beginning of ejaculation

Ejaculation duration (ED) – Time ranging between the beginning of the ejaculation and the end of ejaculation (ended genital contact).

Microscopy analysis

Digital images were obtained using a Zeiss AxioImager M2 upright microscope equipped with a digital CCD camera (Zeiss AxioCam MRm) and an EC Plan-Neofluar 20x/0.5 objective. Images were captured in brightfield by performing a Z-stack (30 Z planes spaced 1um of each other) and XY-tiling acquisition (4 x 3 images) of the brain areas of interest. The number of immunoreactive cells was analysed in coronal sections containing three different brain regions, Arcuate Nucleus of the hypothalamus (ARN) between 1.22 and 2.80 mm caudal from Bregma, Medial Preoptic area of hypothalamus (MPA) between 0.74 rostral and 0.70 mm caudal of Bregma and the Paraventricular Nucleus of the hypothalamus (PVN) between 0.22 and 1.22 mm caudal from Bregma according to stereotaxic coordinates (Franklin and Paxinos, 2008). For each nucleus, we analysed four different images representing anterior, medial and posterior part of each nucleus.

From the XY images obtained, we used ImageJ (http://imagej.nih.gov/ij/) software to analyse the number of immunoreactive cells. A 500 x 700 pixel area (255.86 x 358.24 µm) was defined for ARN and MPA nucleus and to PVN a 500x 600 pixel area (255.86 x 307,06 µm). Selection area was positioned considering pre-defined brain landmarks. The third ventricle and the base of the brain as well the knowledge of the relative position of each nucleus, by analysing the stereotaxic coordinates (Franklin and Paxinos, 2008), were considered as a reference to establish the area to be quantified in the cases of MPA and ARN, the dorsal termination of the third ventricle was the reference to establish the PVN area to be quantified.

Maximum Intensity Projections were generated for each Z-stack of selected regions of interest and the number and average intensity of immunoreactive elements was calculated after segmentation with an adaptive local threshold.

Statistics

To characterise the male sexual behaviour for each strain (PWD, PWK, BL6 and Balbc) we used non-parametric tests, which rely on no particular assumption, since data did not respect normality assumptions (Kolmogorov-Smirnov and Shapiro-Wilk tests), even after applying data transformation (Log transformation, square root transformation and reciprocal transformation).

Non- parametric version of one-way ANOVA (Kruskal-Wallis test) was used to for the comparison between K independent samples followed by Dunn's multiple comparisons test for multiple pairwise comparisons using a Bonferroni- Dunn's 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 deviation (X ± SD). For two independent samples the non-parametric Mann-whitney tests was applies. These statistical analyses were performed using GraphPad Prism® software. Interactions between two categorical variables were analysed by conducting a chi-square and/or a Fisher exact test to those case that were violated chi-square assumptions.

Images resulting from the immunostaining protocol were analysed by doing a two way ANOVA. Some cell counts respected normality assumptions (Kolmogorov-Smirnov and Shapiro- Wilk tests), while in other cases we applied data transformation (Log transformation and Square root transformation) making successfully the data fitting into a normal distribution. These two analyses were performed using IBM SPSS statistics® software (version 21).

Results

1. Male sexual behaviour of musculus and domesticus mice

We started by characterising and comparing the sexual behaviour of males from the two subspecies of house mouse: *Mus musculus domesticus* and *Mus musculus musculus*. For that we chose two representatives of each subspecies: domesticus- C57BL/6J (BL6), Balb/Cj (Balbc); musculus- PWD/PhJ (PWD) and PWK/PhJ (PWK). BL6 and Balbc, two classical laboratory strains of mice routinely used for research, are derived from the three different subspecies of house mouse: domesticus, musculus and castaneus [51]. However, they are predominantly domesticus and hence have been used as a representative of this subspecies [53].

We started by performing a preliminary analysis of the sexual behaviour across the four strains of male mice to get a sense if the behaviour was (or not) similar within strains of the same subspecies and between the two subspecies. We started by detecting the time of occurrence of three behavioural events that could give us a rough idea of the behavioural dynamics: the time of the beginning of each mount (which, besides giving us the mount frequency, also gives us the total number of mounts that were needed to reach ejaculation); the time when the male ejaculated; and the first mount after reaching ejaculation (which is an indirect measure of the end of the refractory period). In Figure 1 we plotted the time of occurrence for each behaviour for each strain. The plots were aligned to the beginning of the trial (female entering in male box). Visual inspection alone of these plots indicates that different strains behave differently and that sexual behaviour is more similar between animals of the same subspecies, but different across subspecies, at least for some of the measures. On Figure 2 we plot the total number of mounts for each strain and the time needed to reach ejaculation. The number of mounts needed to reach ejaculation and the time to ejaculate is statistically the same within subspecies. However, the number of mounts to ejaculate is also similar between PWK/PWD and Balbc and the latency to ejaculate is the same between PWK and B6/Balbc.

Finally, in terms of recovery of arousal after ejaculation (Figure 3), while the percentage of domesticus animals that recovers is zero or very low, musculus animals have a statistically significant higher probability (Pearson chi-square test; $p<0,05$) of engaging in sexual behaviour within two hours after ejaculation (PWK 80% that recovered from the PERP in a 120 minutes period after ejaculation, followed by PWD males 44,4% and of BL6 males 10%; In the case of Balbc males none of the males recovered from PERP). Statistically, PWK and PWD males did not seem to differ in the capacity to recover from PERP, however BL6 and Balbc exhibit a significant difference from PWK strain. However, within the animals that do recover, the time for the first mount after ejaculation is the same across the four strains (Figure 3).

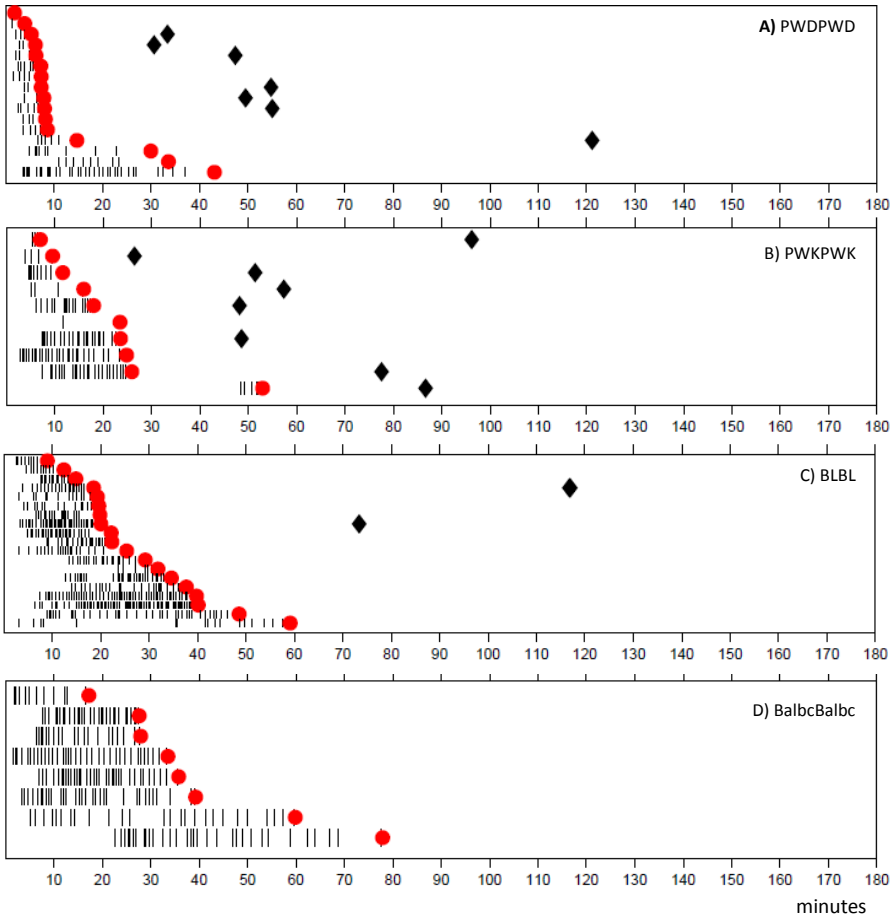

Figure1. Within strain male sexual behaviour. Raster plots are aligned to the insertion of the female in the behavioural paradigm and depict mount events (black ticks), ejaculation (red circles) and the first mount event after ejaculation (black diamonds) for A) PWD male x PWD female, N=17; B) PWK male x PWK female, N= 10; C) BL male x BL female, N=19; D) Balbc male x Balbc female, N=8).

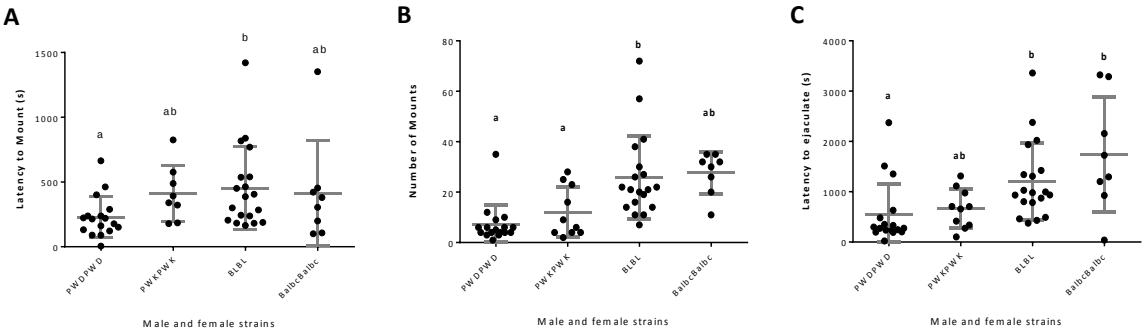

Figure2. Male sexual behaviour across different male and female pairs of the same subspecies. A) Latency to mount in seconds (X ± SD PWDPWD = 228.1 ± 158.2, N=17; X ± SD PWKPWK = 413.4 ± 214.9, N=8; X ± SD BLBL = 473.2 ± 331.3, N=17; X ± SD BalbcBalbc = 414.9 ± 402.4, N=8). B) Number of mounts (X ± SD PWDPWD = 7.176 ± 7.675, N=17; X ± SD PWKPWK = 12.10 ± 9.994, N=10; X ± SD BLBL = 25.74 ± 16.38, N=19; X ± SD BalbcBalbc = 27.63 ± 8.366, N=8). C) Latency to ejaculate in seconds (X ± SD PWDPWD = 541.3 ± 620.2, N= 17; X ± SD PWKPWK = 660.4 ± 388.5, N=10; X ± SD BLBL = 1203 ± 759.0, N=19; X ± SD BalbcBalbc = 1744 ± 1141, N=8). One way ANOVA Kruskal-Wallis test, $p<0.05$; Different letters (a, b, c) indicate a $p<0.05$ obtained by Bonferroni-Dunn's multiple comparisons test.

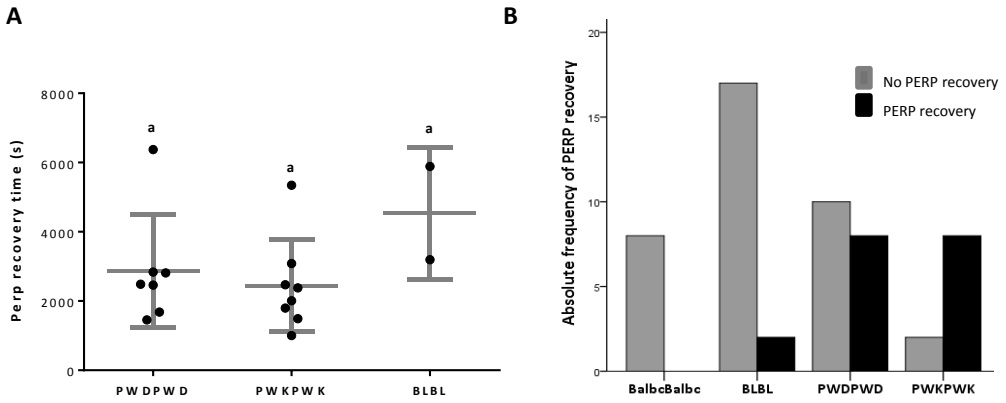

Figure 3. A) Post ejaculatory refractory period in seconds for male mice to start mount females of the same subspecies (X ± SD PWDPWD = 2872 ± 1634, N=7; X ± SD PWKPWK = 2447 ± 1332, N=8; X ± SD BLBL = 4540 ± 1904, N=2). One way ANOVA Kruskal-Wallis test, $p>0.05$; Equal letter (a) indicate a $p>0.05$ obtained by Bonferroni-Dunn's multiple comparisons test. B) Frequency of males that restarted to mount after an ejaculation with females of the same subspecies. Pearson Qui-square $p<0,05$ (association between strains and probability to recover from PERP)

To further investigate the sexual behaviour of the two subspecies, we analysed several other behavioural measurements (total mount duration, average mount duration, number of mount attempts and inter–mount interval), Figure 4. A similar image is observed in terms of the other behavioural variables, where animals of the same subspecies are more similar to each other, but also some similarities between members of the two subspecies. The only clear exception is related to the total amount of mounting, where PWK and PWD are similar and both different from B6 and Balbc. This is, the musculus' strains seem to need less amount of sensory genital stimulation in order to reach ejaculation.

In conclusion (but not forgetting that we do not have a clean separation between the two subspecies) the two strains of each subspecies seem to behave similarly, but for some behavioural measures there are also similarities across subspecies. The results also seem to support that PWD and PWK strains needed less interaction with the female to reach ejaculation. Altogether, these results suggest that there is a difference between subspecies/strains that have different genetic backgrounds leading to different sexual behaviour output. *Musculus* strains seem to need less sexual interaction to reach ejaculation as well as demonstrate a higher percentage of males that recovered from the PERP.

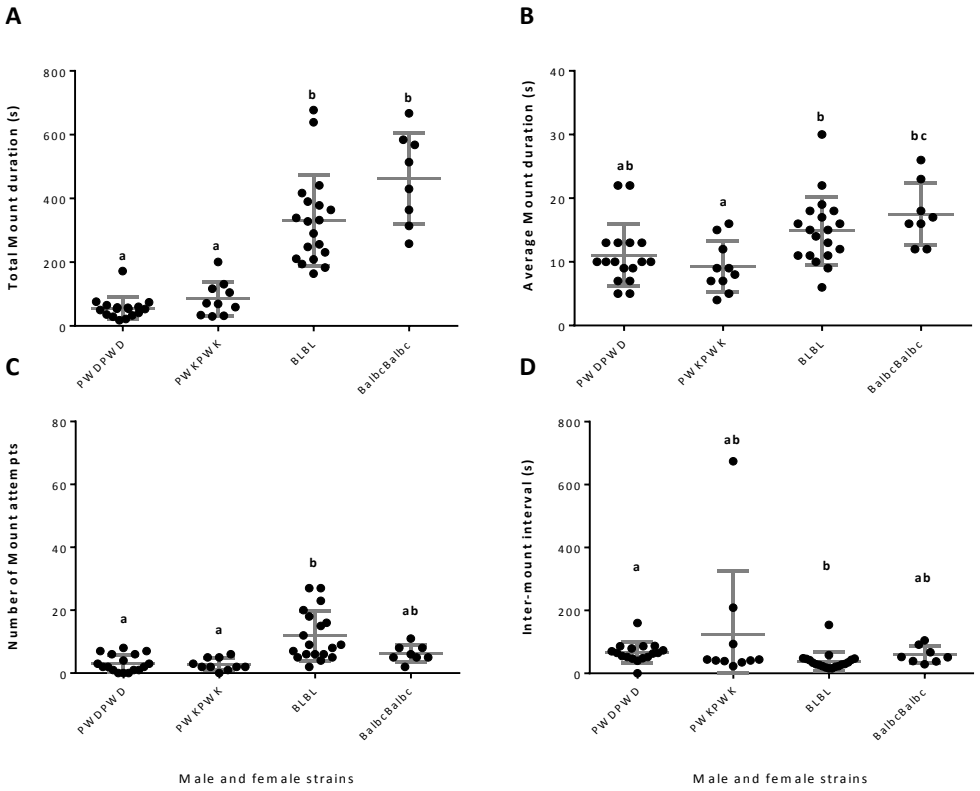

Figure 4. Male sexual traits across different male and female pairs of the same subspecies. A) Total mount duration in seconds (X ± SD PWDPWD = 56.00 ± 34.29, N=17; X ± SD PWKPWK = 84.90 ± 54.32, N=10; X ± SD BLBL = 331.1 ± 142.0, N=19; X ± SD BalbcBalbc = 462.4 ± 143.9, N=8). B) Average mount duration in seconds (X ± SD PWDPWD = 11.06 ± 4.841, N=17; X ± SD PWKPWK = 9.200± 3.994, N=10; X ± SD BLBL = 14.89 ± 5.363, N=19; X ± SD BalbcBalbc = 17.50 ± 4.899, N=8). C) Number of mount attempts (X ± SD PWDPWD = 3.118 ± 2.713, N=17; X ± SD PWKPWK = 2.800 ± 1.932, N=10; X ± SD BLBL = 11.84 ± 7.904, N=19; X ± SD BalbcBalbc = 6.250 ± 2.712, N=8). D) Inter-mount interval in seconds (X ± SD PWDPWD = 66.35 ± 32.16, N=17; X ± SD PWKPWK = 124.3 ± 200.7, N=10; X ± SD BLBL = 38.89 ± 30.36, N=19; X ± SD BalbcBalbc = 59.00 ± 26.88, N=8). One way ANOVA Kruskal-Wallis test, $p<0.05$; Different letters (a, b, c) indicate a $p<0.05$ obtained by Bonferroni-Dunn's multiple comparisons test.

2. Female role in male sexual behaviour

Having in mind that sexual behaviour in mammals involves the interaction between two individuals of opposite sex it is necessary to incorporate the role of the female while examining male sexual behaviour. To test if the differences in male sexual behaviour are due to male behaviour or to the interaction of the two individuals, we exposed males to females of a different strain, to investigate how the interaction with the female influences the male behavioural output. This way we could measure how the differences registered in the previous tests were established only by the male and not by the result of the interaction of male and female.

We choose males from three strains of mice, PWD, PWK and Bl6, and we exposed each strain to females of the other strains: PWK males were exposed to PWD and BL6 females (PWKPWD and PWKBL), PWD males were exposed to BL6 and PWK females (PWDBL and PWDPWK) and BL6 males were exposed to PWD females (BLPWD) (Note: not all combinations were possible, either because animals were not available or because the pair did not reach ejaculation). The behaviour was compared to the behaviour of males that interacted with females from their own strain: In total we had eight different combinations to compare. The combinations of males with females from the same subspecies were the ones used in experiment 1.

Regarding Bl6 males interactions, (BLBL and BLPWD) noticeable differences between conditions arise from the analysis of the raster plot (Figure 5).

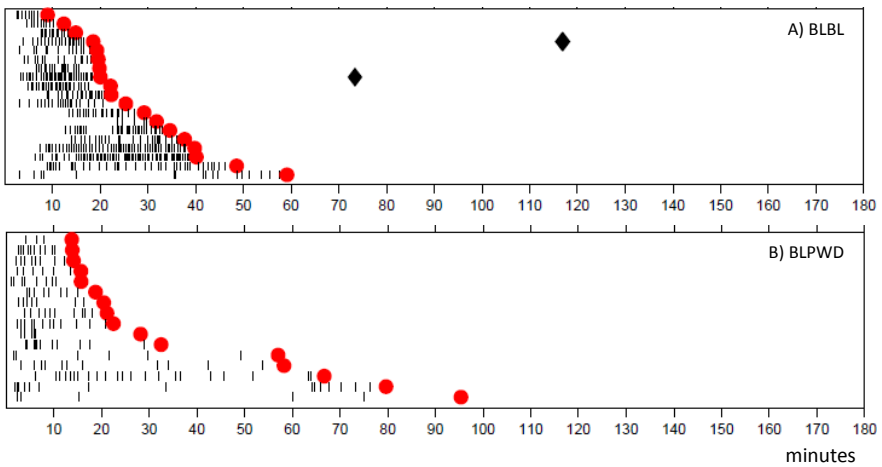

Figure5. *Mus musculus domesticus* BL6 male sexual behaviour with different female strains. Raster plots are aligned to the insertion of the female in the behavioural paradigm and depict mount events (black ticks), ejaculation (red circles) and the first mount event after ejaculation (black diamonds) for A) BL male x BL female, N=19; B) BL male x PWD female, N=16.

Bl6 males when interacting with PWD females decrease significantly the time needed to start mounting the female (latency to mount, Figure 6A) as well as the number of mounts that the males need to reach ejaculation (Figure 6B). Even though when compared the time elapsed between the first mount and ejaculation there is no difference when compared to a condition where BL6 males are interacting with a BL6 female (Figure 6 C).

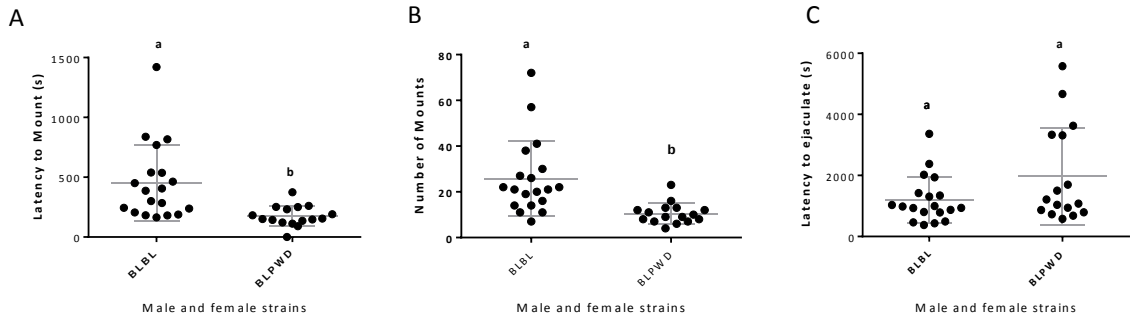

Figure 6. BL male sexual behaviour with different female subspecies. A) Latency to mount in seconds (X ± SE: BLBL = 453.3 ± 319.8, N=19; X ± SD BLPWD = 174.8 ± 85.9, N=16). B) Number of mounts (X ± SD BLBL = 25.74 ± 16.38, N=19; X ± SD BLPWD = 10.5 ± 4.53, N=16). C) Latency to ejaculate in seconds (X ± SD BLBL = 1203.1 ± 759.0 N= 19; X ± SD BLPWD = 1976.7 ± 1592.9, N=16). Different letters (a, b) indicate a $p<0.05$ obtained by the non-parametric Mann Whitney test.

Raster plot (Figure 5) analysis shows that the interaction with a PWD female affected the Bl6 male sexual behaviour pattern. When examining other behavioural parameters (Figure 7) we see that the interaction with a female from a different subspecies, leads to decrease on the total mount duration and on the average mount duration. Contrarily there is a significant increase on the number of mount attempts as well as in the inter-mount interval. Latency to ejaculate was the only parameter that showed to not be affected by the interaction with females from different subspecies. This result leads to argue that BL6 males seem to be highly affected by the interaction with a PWD female. BL males interacting with PWD females need less amount of genital stimulations.

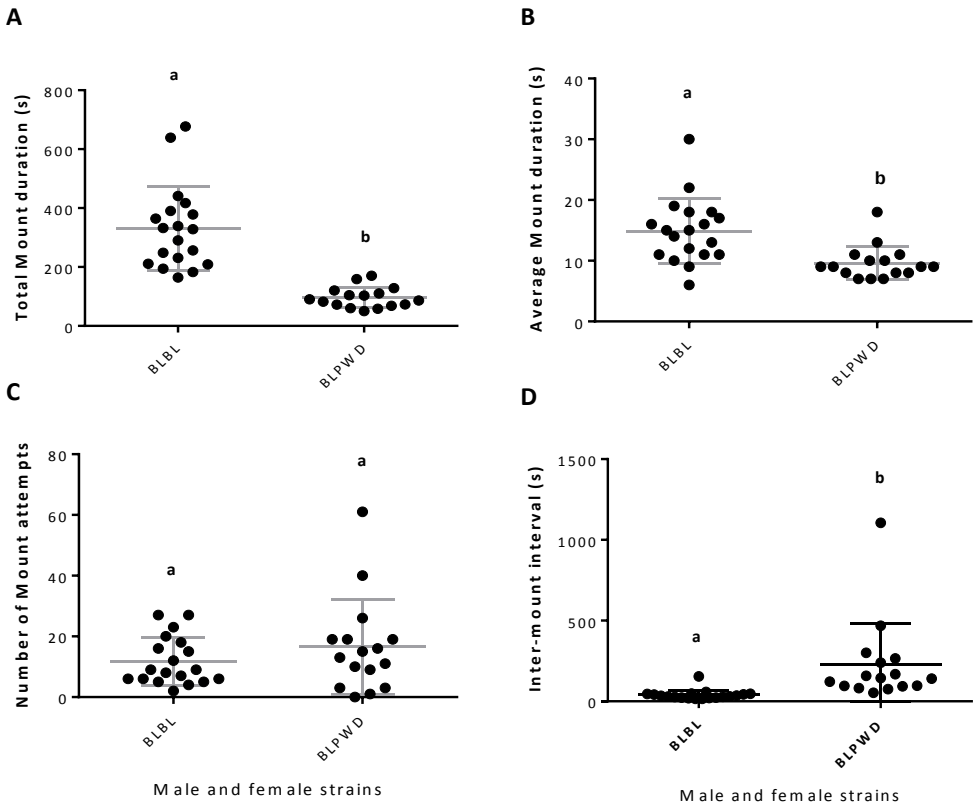

Figure 7. BL6 male sexual traits with different female subspecies. A) Total mount duration in seconds (X ± SE: BLBL = 331.1 ± 142.0, N=19; X ± SD BLPWD = 95.8 ± 35.1, N=16). B) Average mount duration in seconds (X ± SD BLBL = 14.9 ± 5.4, N=19; X ± SD BLPWD = 9.6 ± 2.8, N=16). C) Number of mount attempts (X ± SD BLBL = 11.8 ± 7.9 N= 19; X ± SD BLPWD = 16.6 ± 15.6, N=16). D) Inter-mount interval in seconds (X ± SD BLBL = 38.9 ± 30.4 N= 19; X ± SD BLPWD = 225.2 ± 257.9, N=16). Different letters (a, b) indicate a $p<0.05$ obtained by the non-parametric Mann Whitney test.

The interaction of PWD males with females from the PWK and BL6 strains also showed to have an effect of the male sexual behaviour. When studying the results from the raster plot (Figure 8) we see that when PWD males are exposed to Bl6 females, they show a significant higher number mounts before reaching ejaculation (Figure 9B) along with a bigger latency to start mounting the female (Figure 9A); different from what happens with a PWK female. Interacting with a PWK female did not show to have an effect in both parameters.

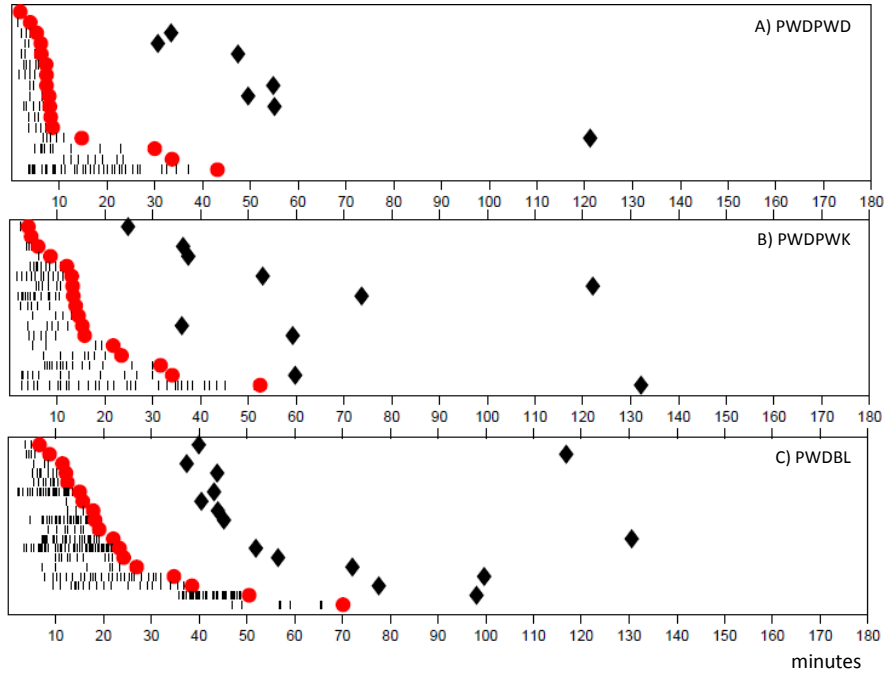

Figure 8. *Mus musculus musculus* PWD male sexual behaviour with different female strains. Raster plots are aligned to the insertion of the female in the behavioural paradigm and depict mount events (black ticks), ejaculation (red circles) and the first mount event after ejaculation (black diamonds) for A) PWD male x PWD female, N=17; B) PWD male x PWK female, N=17; C) PWD male x BL female, N=18.

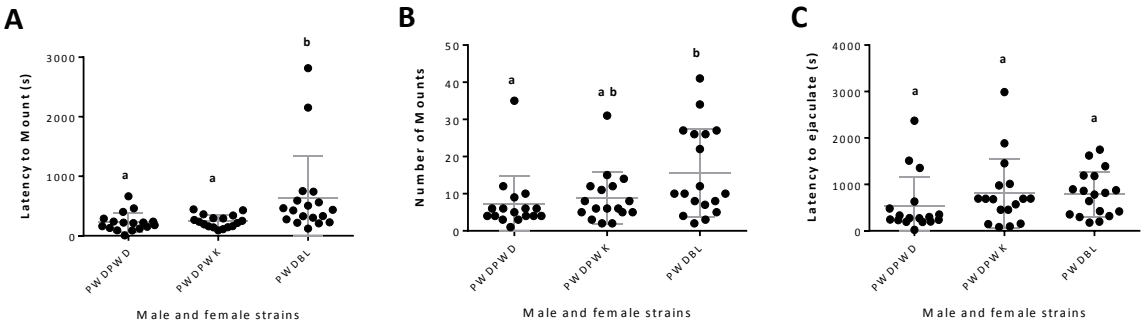

Figure 9. PWD male sexual behaviour with different female subspecies. A) Latency to mount in seconds (X ± SD PWDPWD = 228.1 ± 158.2, N=17; X ± SD PWDPWK = 243.2 ± 106.8, N=17; X ± SD PWDBL = 636.6 ± 704.2 , N=18). B) Number of mounts (X ± SD PWDPWD = 7.176 ± 7.675, N=17; X ± SD PWDPWK = 8.882 ± 6.972, N=17; X ± SD PWDBL = 15.67 ± 11.85, N=18). C) Latency to ejaculate in seconds (X ± SD PWDPWD = 541.3 ± 620.2, N=17; X ± SD PWDPWK = 807.7 ± 736.7, N=17; X ± SD PWDBL = 788.7 ± 483.9, N=18). One way ANOVA Kruskal-Wallis test, $p<0.05$ for A) and B); Different letters (a, b) indicate a $p<0.05$ obtained by Bonferroni-Dunn's multiple comparisons test.

Equally from what happened with BL6 males, the interaction with different females did not change the time that males took to ejaculate after starting mounting the female (Figure 9C). The PWD capacity of recovery from PERP seems to be affected by the female (Pearson chi-square test; p<0,05) with whom they interacted with (Figure 10). Interacting with Bl6 females leads to 83,3% of individuals recovering from PERP in the 120 minutes period, a statistically significantly higher percentage than in PWDPWD condition, that only exhibited 44,4% of males recovering from PERP. Further analysis showed that, PWD males only seem to exhibit a shift in his sexual behaviour when interacting with a BL6/*M.domesticus* strain. Parameters such as the total mount duration, the number mounts and the numbers of mount attempts are significantly increased in relationship to the basal condition PWDPWD (Figure 11). Even though, these results seem to argue a different effect caused by *M.musculus* versus *M.domesticus* on the sexual behaviour of PWD male, we need to be careful in attributing this effect solely to the subspecies difference. BL6 females are larger than PWK females and we did not control for weight. Larger females can be harder to grasp and lead to the observed changes in sexual behaviour. Future experiments are needed to tease the role of the female weight.

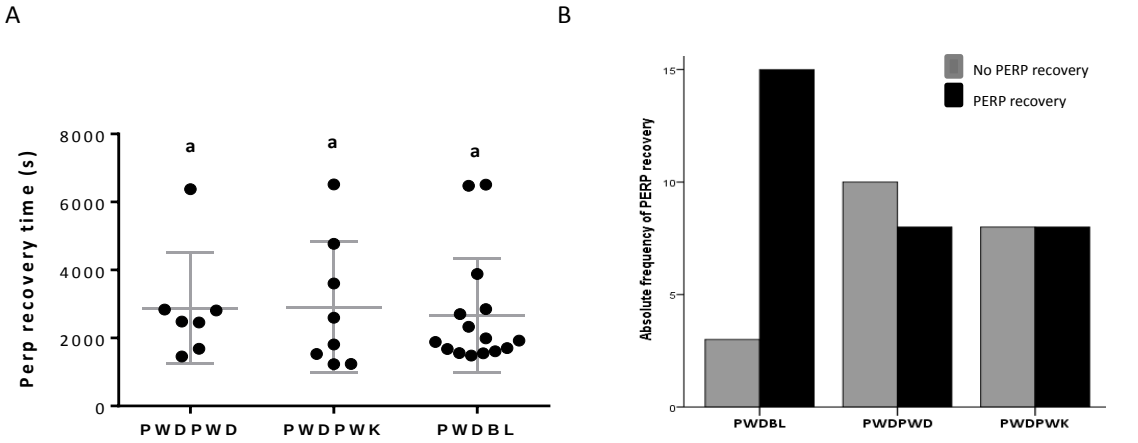

Figure 10. A) Post ejaculatory refractory period in seconds for PWD male mice to start mount females of different subspecies (X ± SD PWDPWD = 2872 ± 1634, N=7; X ± SD PWDPWK = 2912 ± 1919, N=8; X ± SD PWDBL = 2674 ± 1678, N=15). One way ANOVA Kruskal-Wallis test, p>0.05; Equal letter (a) indicate a p>0.05 obtained by Bonferroni-Dunn's multiple comparisons test. B) Frequency of PWD males that restarted to mount after an ejaculation with females of different subspecies. Pearson Qui-square p<0,05 (no association between strains and probability to recover from PERP).

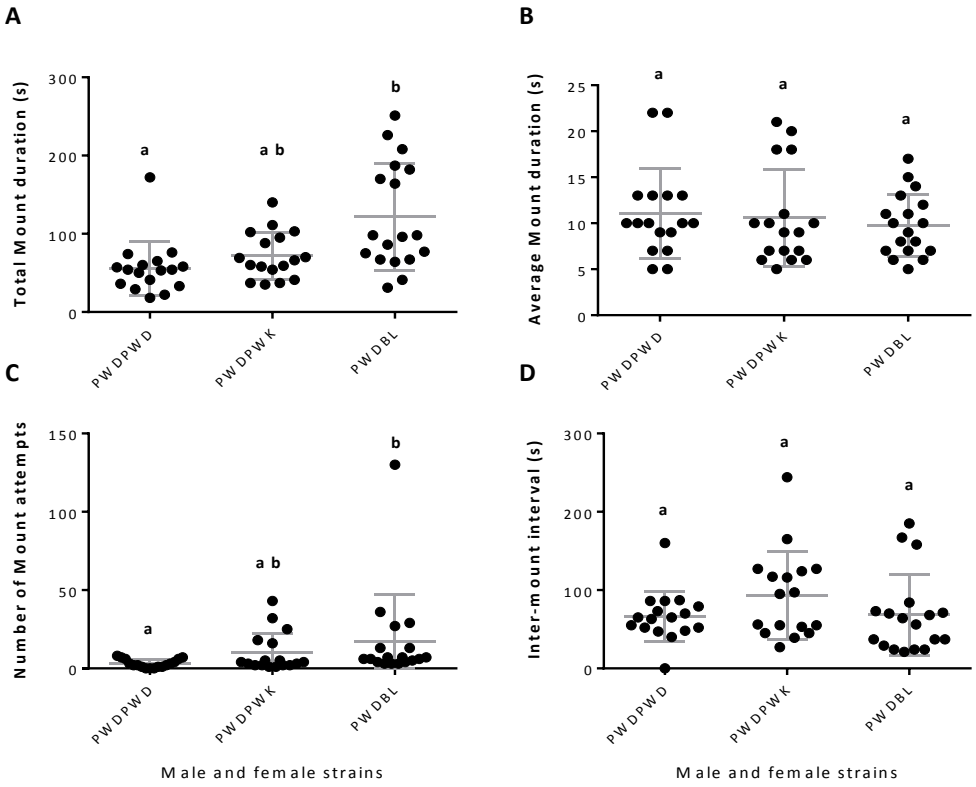

Figure 11. PWD male sexual traits with different female subspecies. A) Total mount duration in seconds (X ± SD PWDPWD = 56.00 ± 34.29, N=17; X ± SD PWDPWK = 72.06 ± 30.06, N=17; X ± SD PWDBL = 121.6 ± 67.89, N=18). B) Average mount duration in seconds (X ± SD PWDPWD = 11.06 ± 4.841, N=17; X ± SD PWDPWK = 10.59 ± 5.280, N=17; X ± SD PWDBL = 9.778 ± 3.405, N=18). C) Number of mount attempts (X ± SD PWDPWD = 3.118 ± 2.713, N=17; X ± SD PWDPWK = 9.882 ± 12.59, N=17; X ± SD PWDBL = 17.17 ± 29.84, N=18). D) Inter-mount interval in seconds (X ± SD PWDPWD = 66.35 ± 32.16, N=17; X ± SD PWDPWK = 93.35 ± 55.97, N=17; X ± SD PWDBL = 68.28 ± 51.14, N=18) One way ANOVA Kruskal-Wallis test, $p<0.05$ for A) and C); Different letters (a, b) indicate a $p<0.05$ obtained by Bonferroni-Dunn's multiple comparisons test.

Interestingly, in the case of PWK males we do not see an effect of the subspecies of the interacting female. Analysis of the raster plot (Supplementary figure 1) as well all the other parameters (Supplementary figures 2, 3 and 4) show no statistical significant difference in the sexual behaviour of PWK males interacting with PWD or BL6 females. Once again, BL6 females are also larger than PWD females and the size difference was not controlled.

The sexual pairs involving BL6 and PWD males were the ones where different females seem to have a higher impact on the male's sexual behaviour. Therefore, we zoomed into the behaviour of these pairs for further analysis and we run statistical tests to four different combinations (PWDPWD, PWDBL, BLBL and BLPWD) involving PWD and BL6 males and females

The analysis of number of mounts, latency for the first mount and latency to ejaculate, shows that only in the case of PWDPWD vs BLPWD there is a significant difference in the time needed to ejaculate: BL6 males interacting with PWD females do not seem to have a disruption of his basal condition (Figure 12).

The interaction between Bl6 males with PWD females (BLPWD) seems to cause a greater impact on BL6 male behaviour, being this condition statistically different from the one when BL6 males are exposed to Bl6 females (as previously shown). For parameters such as, average mount duration, total mount duration, the number of mounts and inter-mount interval (Figure 13), BLPWD condition exhibit significant differences from BL6BL6 condition but, interestingly a non-significant difference from PWDPWD condition. With the exception of an increase in of inter-mount interval (Figure13 D), we observed a decrease on the mean value of BLPWD when compared with BL6Bl6 condition. In regard of the interaction of PWD males with Bl6 females (PWDBL) we also saw a modification in male PWD sexual behaviour. As anteriorly reported, total mount duration, number of mounts and number of mount attempts are significantly different from those showed by PWDPWD condition, and only maintaining statistically significant differences in total mount duration when compared with BL6BL6 condition (Figure 13.). Recovery from PERP seems to be mainly established by the male: PWD males seem to recover with higher probability independently of being with a PWD or a Bl6 female (table). From all the conditions the one that shows a higher percentage of individuals that recovered from PERP in the period of 120 minutes after ejaculation was the PWDBL (83,3%) followed by PWDPWD (44,4%), BLBL (10,5%) and at last BLPWD, with the males no recovering from PERP. Statistically we had a significant difference between BLBL/BLPWD from PWDBL/PWDPWD conditions (Figure 14). The comparison from BLPWD vs PWDPWD and PWDBL vs BLBL showed that in the case of BLPWD, the females had a higher impacting in affecting the sexual behaviour of the male.

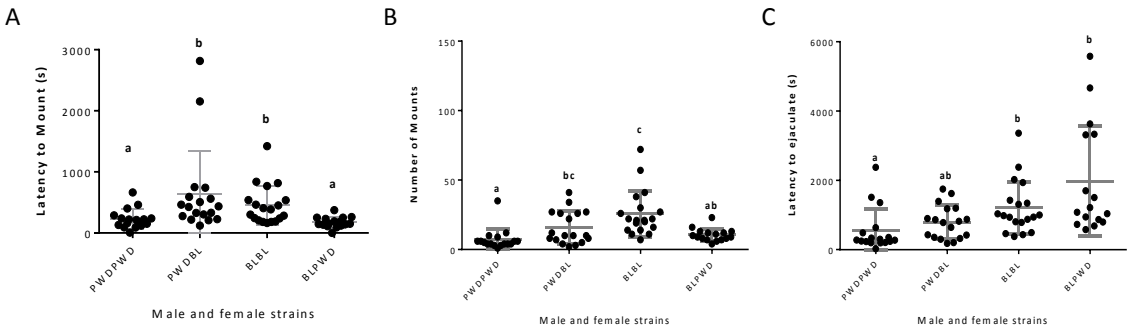

Figure 12. PWD and BL male sexual behaviour with PWD and BL females. A) Latency to mount in seconds (X ± SD PWDPWD = 228.1 ± 158.2, N=17; X ± SD PWDBL = 636.6 ± 704.2, N=18; X ± SD BLBL = 473.2 ± 331.1, N=17; X ± SD BLPWD = 174.8 ± 85.90, N=16). B) Number of mounts (X ± SD PWDPWD = 7.176 ± 7.675, N=17; X ± SD PWDBL = 15.67 ± 11.85, N=18; X ± SD BLBL = 25.74 ± 16.38, N=19; X ± SD BLPWD = 10.50 ± 4.53, N=16). C) Latency to ejaculate in seconds (X ± SD PWDPWD = 541.3 ± 620.2, N=17; X ± SD PWDBL = 788.7 ± 483.9, N=18; X ± SD BLBL = 1203 ± 759.0, N=19; X ± SD BLPWD = 1977 ± 1593, N=16). One way ANOVA Kruskal-Wallis test, $p<0.05$; Different letters (a, b, c) indicate a $p<0.05$ obtained by Bonferroni-Dunn's multiple comparisons test.

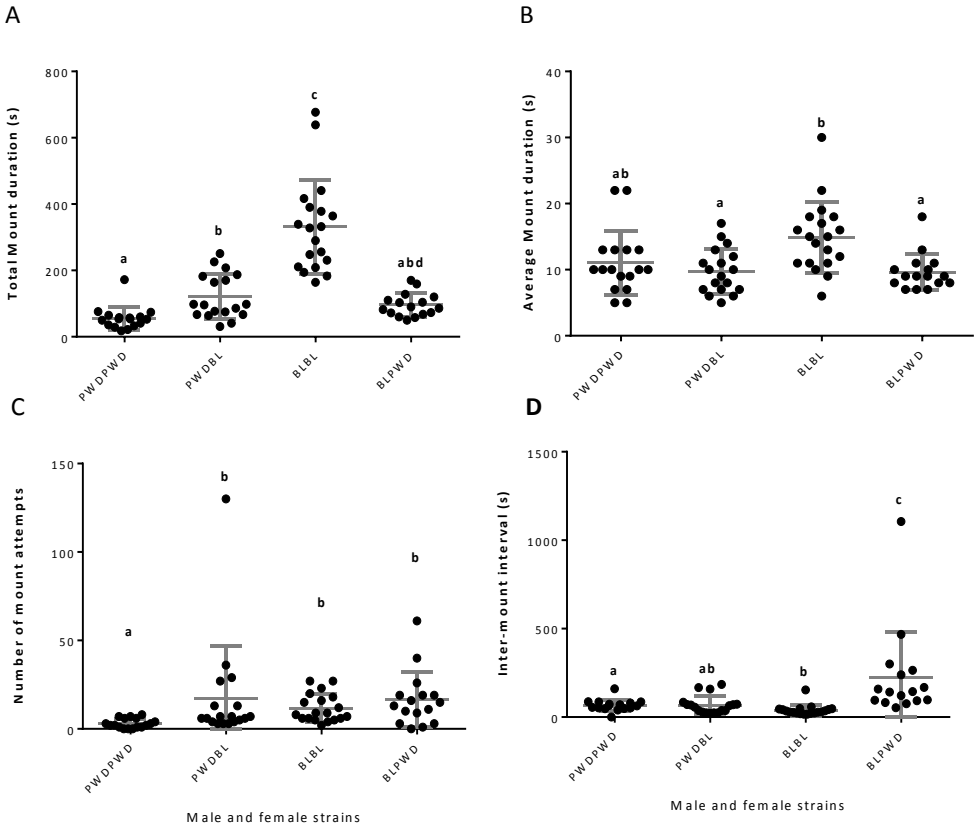

Figure 13. PWD and BL male sexual traits with PWD and BL females. A) Total mount duration in seconds (X ± SD PWDPWD = 56.00 ± 34.29, N=17; X ± SD PWDBL = 121.6 ± 67.89, N=18; X ± SD BLBL = 331.1 ± 95.81, N=19; X ± SD BLPWD = 95.81 ± 35.05, N=16). B) Average mount duration in seconds (X ± SD PWDPWD = 11.06 ± 4.841, N=17; X ± SD PWDBL = 9.78 ± 3.41, N=18; X ± SD BLBL = 14.89 ± 5.37, N=19; X ± SD BLPWD = 9.63 ± 2.78, N=16). C) Number of mount attempts (X ± SD PWDPWD = 3.118 ± 2.713, N=17; X ± SD PWDBL = 17.17 ± 29.84, N=18; X ± SD BLBL = 11.84 ± 7.90, N=19; X ± SD BLPWD = 16.56 ± 15.62, N=16). D) Inter-mount interval in seconds (X ± SD PWDPWD = 66.35 ± 32.16, N=17; X ± SD PWDBL = 68.28 ± 51.14, N=18; X ± SD BLBL = 38.89 ± 30.36, N=19; X ± SD BLPWD = 225.4 ± 257.9, N=16) One way ANOVA Kruskal-Wallis test, p<0.05; Different letters (a, b, c and d) indicate a p<0.05 obtained by Bonferroni-Dunn's multiple comparisons test.

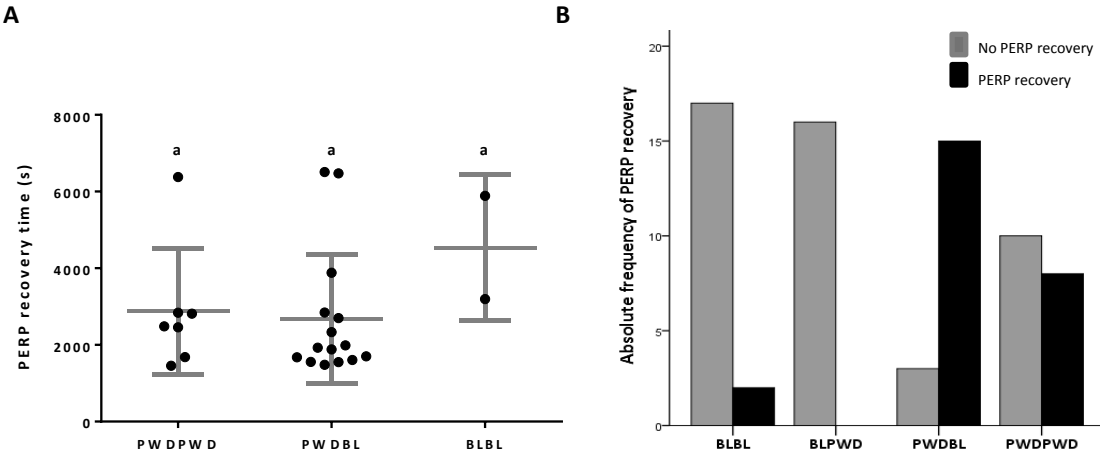

Figure 14. A) Post ejaculatory refractory period in seconds for PWD and BL male mice to start mount PWD and BL females (X ± SD PWDPWD = 2872 ± 1834, N=7; X ± SD PWDBL = 2674 ± 1678, N=15; X ± SD BLBL = 4540 ± 1904, N=2). One way ANOVA Kruskal-Wallis test, p>0.05; Equal letter (a) indicate a p>0.05 obtained by Bonferroni-Dunn's multiple comparisons test. B) Frequency of PWD and BL males that restarted to mount after an ejaculation with PWD and BL females. Pearson Qui-square p<0,05 (association between strains and probability to recover from PERP).

3. STAT 5 activation across males of the different strains

Human studies had previously shown that upon ejaculation men experience a peak of circulating PRL in the blood [26] and it has been hypothesized that the PRL surge is in fact responsible for the establishment of the refractory period experienced upon ejaculation, [33] [34]. However, until today no study has actually established a causal relationship between PRL and the refractory period. Because our different strains of mice exhibit such marked difference in terms of behaviour during the refractory period (musculus recover sexual drive in less than two hours, while domesticus do not), we decided to test if the brain of each mouse could in principle respond to PRL in a different manner. We wanted to investigate how each strain would respond to an artificial intraperitoneal injection of PRL. We investigated PRL activation indirectly using STAT5 and we measured the effect in three brain regions which have already been shown to respond to PRL: Arcuate nucleus of the hypothalamus (ARN), the Medial Preoptic area of the hypothalamus (MPA) and the Paraventricular nucleus of the hypothalamus (PVN). While the ARN would serve as an internal control because it is thought to be involved in the output area that control PRL release and is involved in all behaviors dependent on PRL, both the PVN and MPOA are important nuclei for socio-sexual behaviour. The test was done in males from different mice strains (PWD, PWK, BL and Balbc) since they had shown a differential behavioural output, more specifically a different capacity to recover from the PERP (Figure 3).

Experimentally, we used the Signal Transducer and Activator of Transcription 5 (STAT5) phosphorylation to confirm if the activation of neurons were due to the presence of prolactin, [57] [58]. STAT5 is a protein from the JAK-STAT pathway that is involved in the transduction of PRL signal received by its receptor. Previous studies showed that STAT 5 has a high specificity to the activation of the transducer from PRL [58] being a good way to track the neuronal activation in response of PRL changes. By injecting PRL into circulate (Figure 15). Our prediction was that, if PRL is indeed involved in the behavioural differences, we would see differential activation of STAT5. This would be the first step to then investigate what changes does STAT5 induce in neurons that could lead to changes in sexual behaviour.

For each brain nucleus we compared the number of cells that were activated in each strain, and in each treatment (vehicle versus PRL injection, 2-way ANOVA).

For the three candidate areas we did not observe a significant activation difference that could be explained by the injection of PRL (Figure16 and table 2). ARN and PVN activation results (Figure 16) showed that PWD males have intrinsically higher activation of cells in comparison with BL6 and PWK in the case of the ARN and with PWK in the case of PVN. This activation seen in the vehicle-injected animals is due to baseline, tonic levels of PRL that are always present in animals.

In summary, even though different strains have different resting state of STAT5 activation, the increased STAT5 caused by exogenous PRL is the same across all animals. Hence we cannot establish, with this method, any relationship between the capacity of these three brain regions to respond to PRL and the different behavioural results obtained.

A

Dependent Variable: SQ_cells

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	586,901[a]	7	83,843	16,884	,000
Intercept	2675,292	1	2675,292	538,726	,000
Strains	14,092	3	4,697	,946	,445
Treatment	542,911	1	542,911	109,326	,000
Strains * Treatment	19,657	3	6,552	1,319	,307
Error	69,523	14	4,966		
Total	3472,000	22			
Corrected Total	656,424	21			

a. R Squared = ,894 (Adjusted R Squared = ,841)

B

Dependent Variable: Log_cells

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2,848[a]	7	,407	3,565	,021
Intercept	78,306	1	78,306	686,132	,000
Strains	1,359	3	,453	3,969	,031
Treatment	1,160	1	1,160	10,160	,007
Strains * Treatment	,330	3	,110	,965	,437
Error	1,598	14	,114		
Total	85,438	22			
Corrected Total	4,446	21			

a. R Squared = ,641 (Adjusted R Squared = ,461)

C

Dependent Variable: Cells

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2083299,29[a]	7	297614,184	19,156	,000
Intercept	5115166,815	1	5115166,815	329,241	,000
Strains	269154,455	3	89718,152	5,775	,009
Treatment	1771008,333	1	1771008,333	113,992	,000
Strains * Treatment	94381,152	3	31460,384	2,025	,157
Error	217507,667	14	15536,262		
Total	7648871,000	22			
Corrected Total	2300806,955	21			

a. R Squared = ,905 (Adjusted R Squared = ,858)

Table 2. 2-way ANOVA analysis of strain (Balbc, BL6, PWD and PWK) and treatment (prolactin, vehicle) effect on pSTAT5 cell activation on A) MPOA, B) PVN and C) ARC.

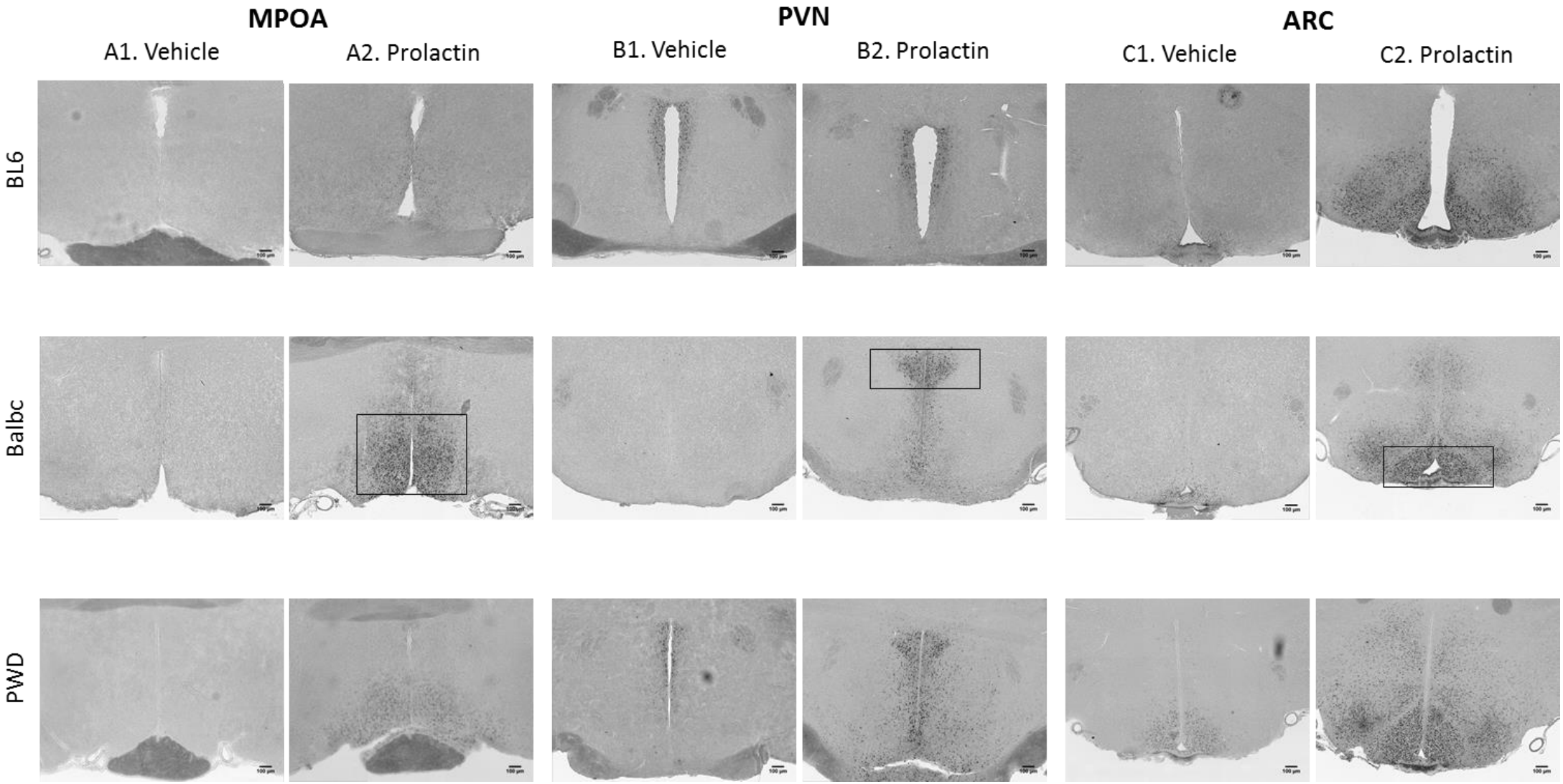
MPOA
PVN
ARC
A1. Vehicle
A2. Prolactin
B1. Vehicle
B2. Prolactin
C1. Vehicle
C2. Prolactin
BL6
Balbc
PWD

Figure 15. Representative brain sections showing pSTAT5 immunohistochemistry in the MPOA, PVN and ARC of different mice strains (rows) treated with vehicle (A1, B1 and C1 columns) or prolactin (A2, B2 and C2 columns). pSTAT5 positive labelling seen as black nuclear staining. Squares in Balbc prolactin treated animals highlight the target areas. (Next page)

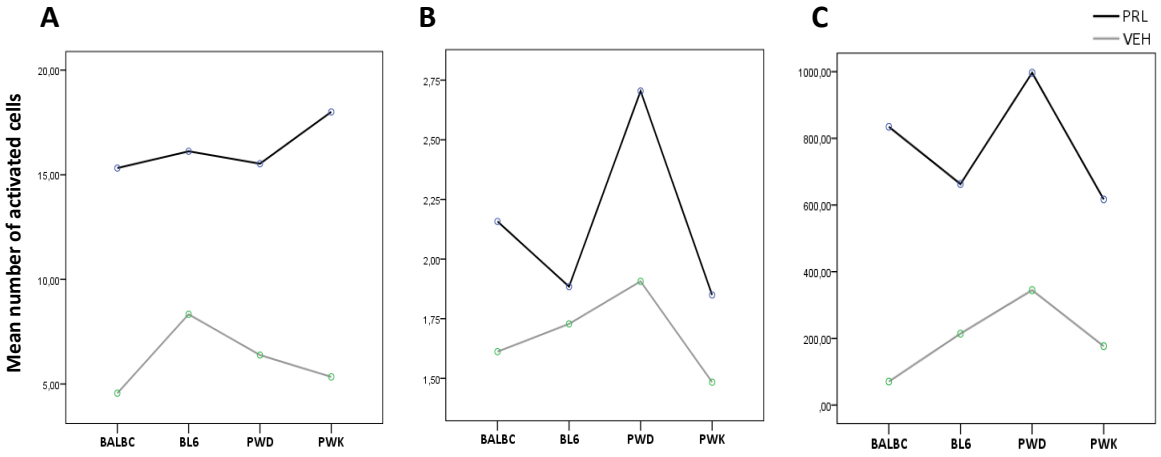

Figure 16. Mean number of pSTAT5 cell activation of A) MPOA, B) PVN and C) ARC of different mice strains treated with vehicle (grey line) or prolactin (black line).

Discussion

In order to know how male mice sexual behaviour varied across two subspecies of mice, *M.domesticus* and *M.musculus* and also how the set of behaviours within sexual behaviour could be shaped by the interaction with a female, we got interesting results.

M. domesticus and *M. musculus* mice in the wild are segregated in different parts of the globe. In Europe, *M. domesticus* is present in Western Europe, whereas *M. musculus* is in Eastern Europe, but still maintaining a hybrid zone [54]. The semi-segregation between these two subspecies of mice during their Evolutionary path could have led to a differentiation in male sexual behaviour, since different optimal strategies might have arisen. By picking four different laboratory strains of mice, we characterised male sexual behaviour and we compared among the subspecies in trial. When comparing the results for the different strains of mice we noted a clear separation between strains from *M. musculus* and *M. domesticus* background. (Figure 1, 2, 3 and 4). No significant differences were seen between strains of the same subspecies only between strains that have a different subspecies background (Figure 1, 2, 3 and 4). When we examined sexual behaviours that are correlated with the interaction that is established with the female (total mount duration, average mount duration, number of mounts, inter-mount interval), we can claim that *M .musculus* males need less interaction to reach ejaculation. It seems that *M. musculus* males need less genital stimulation to reach ejaculation (Figure 1, 2 and 4) and even showing a bigger inter-mount interval (Figure 2) they show a smaller latency to ejaculate as well a higher rate of post-ejaculatory refractory period recovery (Figure 1, 3B).

These results can be interpreted as an ecological/evolutionary consequence of a different distribution in the wild. A different environment could have led to dissimilar sexual optimal strategies. However, other factors have to be considered when analysing the results. Working with laboratory mice strains give us three more factors to consider, genetic background of each strain, laboratory colonization history, such as sexual regimes.

The first mice laboratory strains established in the laboratory happened in an unconstrained way. If we look back, we can see that the first individuals in the lab came up from the idea of creating “fancy” mice, meaning that there was no restrains in the crosses between different subspecies of mice [51]. When we look to the genetic background of the mice strains that we have chosen as our *M. domesticus* representatives (BALB/c and C57BL/6J), we see that their genome is thought to be a mosaic of regions with distinct subspecific origin [53]. Strains, such as C57BL/6J, one of our *M. domesticus* representative showed only to have two-thirds of its genome derived from *M. domesticus* [51]. With this, when looking to our behavioural results we cannot clearly state that the differences that we found, are related to a difference between *M. domesticus* and *M. musculus* or between a mosaic genome and *M.*

musculus. This happens because we do not know if the difference encoded in the *M .domesticus* background or not. This problem does not happen with *M. domesticus* strains, since these strains are one hundred percent *M. musculus* [55].

One of the aspects of mice sexual behaviour is multiple mating. Studies show that in wild mice multiple paternity occurs frequently [59]. A female, by accepting to copulate with more than one male, leads to a position where sperm competition is a sexual selection mechanism. Studies had shown that mice under different sperm competition environments tend to adopt better behavioural strategies [41] [46]. Under these circumstances, male behaviour tends to adopt a strategy with less number of intromissions, less time to ejaculate (latency to ejaculate) and also a smaller post-ejaculatory period (PERP) [46]. Laboratory mice strains oppositely with what happen in the wild are maintained under an inbred regime where breeding pairs are formed from brother and sister individuals, taking out the role of sperm competition in shaping male sexual behaviour.

By being, both subspecies, under a regime of pair crosses, where sperm competition do not exert a role, we could think that we should see differences between the two subspecies since both are under the same regime. However if we take in consideration the number of years since *M. domesticus* strains and *M. musculus* strains are under laboratory conditions we see that *M. domesticus* have at least more 42 years under this conditions than *M. musculus* strains.. The higher number of inbred/pair breedings that *M .domesticus* gone throw could have promoted an adaptation to an environment without sperm competition and yet not visible in *M. musculus strains*

When we look to *M. musculus* strains sexual behaviour (Figure 1, 2, 3 and 4) in comparison to *M. domesticus* strains we can hypothesise that, *M. musculus* were under a sperm competition environment or they still show a sexual behaviour output similar to the wild due to less time under a laboratory regime.

Sexual behaviour supposes the interaction between a male and a female, by this, it is acceptable to think that both male and female influence each other behaviour. In mice sexual behaviour it is known that *M. musculus* females have mate preference for males that are phenotypically similar [54]. Having this in mind we went to test how females could be shaping male sexual behaviour. We conducted a test where different males were exposed to females of the different subspecies and measured how the sexual behaviour parameters were different from a situation where we had an interaction between two individuals from the same subspecies.

The results obtained show that, with a higher preponderance, *M. musculus* females affect considerably the sexual behaviour of *M.domesticus* males. When interacting with *M. musculus* females, *M. domesticus* males show a significant and drastic reduction in their necessity to interact with the female to reach ejaculation (Figure 5, 6

and 7) and by this a less necessity of genital interaction. The interaction between M.musculos females and M.domesticus males seem to make the later one resemble M.musculos males. Therefore, we compared these results with the interactions between M.musculos couples. In three parameters (number of mounts, total mount duration and average mount duration) *M.domesticus* males became comparable with the interaction between two *M.musculus* individuals (Figure 12, 13 and 14). When we observe the opposite situation, having *M. musculus* males interacting with *M. domesticus* females, females' factor seem not to be so profound in *M. domesticus* male sexual behaviour. For one of the two *M. musculus* analysed, the exposure of a *M. domesticus* did not have any effect on male sexual behaviour (Supplementary figure 1, 2 3 and 4).

It seems that *M. musculus* females have a pivot role on shaping male sexual behaviour. When interacting with *M. domesticus* males, *M. musculus* females seem to lead to a more rewarding copula causing a reduction in the number of interactions. As an alternative although not mutually exclusive explanation, M. musculus females may cause a higher sexual arousal in males leading to a lower necessity of genital stimulation to males reach ejaculation. We cannot roll down if this fact is due to a different subspecies origin. *M.domesticus* females seem to have a slight role in male sexual behaviour, however we have to consider the fact that this two subspecies have some physiological differences. M. domesticus individuals are bigger than *M. musculus* individuals what makes harder to male to exhibit the proper sexual behaviours such as mounting the female.

One the behaviours that we analysed was the capability that the two subspecies had to recover from the post-ejaculatory refractory period (PERP). Across different species of rodents we see PERP ranging from minutes to days [8], a phenomenon that still today is not really well understood. In sperm competition environments rodent species tend to exhibit shorter PERP to achieve a higher number of ejaculations in a certain period of time [41 46]. Studies show that under this environment, males allocate less sperm per ejaculation in a way to maximise the number of copulas and maximise the probability to sire more offspring [41 46]. When we see the results obtained for the different subspecies we clearly see the separation between M. musculus and M. domesticus strains. *M. musculus* males have a higher rate of recover from PERP in 120 minutes than *M. domesticus* males. Again, this result can be a result of *M. musculus* males still maintaining a sexual behaviour more similar from what to be expected in wild conditions. When we search for the effect of females in this phenotype we only see a significant result in one of the strains of *M. musculus* males where *M.musculus* males showed to have a higher rate of recovery when interacting with *M. domesticus* females. This result can be a reflection of *M.domesticus* females being less rewarding to *M.musculus* males, leading to a quicker recovery from PERP. When we look to the individuals that recovered from PERP they did not showed significant differences in the

recovery time (Figure 1. 3A). Still, we have to have in mind that we are talking about a comparison done with only two *M. domesticus* males against fifthteen *M. musculus* males (Figure 3A).

Even though individuals from both subspecies showed to have the capability, in the same amount of time, to recover from PERP we tried to see if there are some reasons that can lead to so dissimilar percentages of recovery in the subspecies.

On this past decade, Prolactin (PRL) is being involved in the establishment and maintenance of PERP [26 32 33]. Studies show that upon ejaculation men experience a surge of circulating PRL and that this surge last per 60 minute period. Previous studies in mice also reported a release of PRL upon ejaculation [36]. Since the two different subspecies of mice showed so dissimilar capacities to recover from PERP we went looking for three brain areas and wondered if there was a differential response in the brain to an increase of circulating in the blood that could correlated with the capacity of those males to recover from PERP. We picked three different brain areas that have been related with areas that are activated during sexual behaviour [4]. The method that we employed did not show strain differences in response to an increase of Prolactin, for all three areas we see a similar activation across both subspecies.

With this study we showed that there are differences in male sexual behaviour, but unfortunately we could not determine if those differences arise from the fact that this different strains fit in different subspecies of mice. *M. musculus* showed to drastically change *M. domesticus* behaviour making those males necessity less genital stimulation to reach ejaculation (quality versus quantity) and finally with the employed method we could not give more insight about the Prolactin role in establishing or maintaining PERP. Further studies have to be done to clarify the differences between mice subspecies, and only then start addressing the exact role of female in male sexual behaviour output. Blood analysis should be conducted in order to see if upon ejaculation the different strains have different levels of circulating prolactin.

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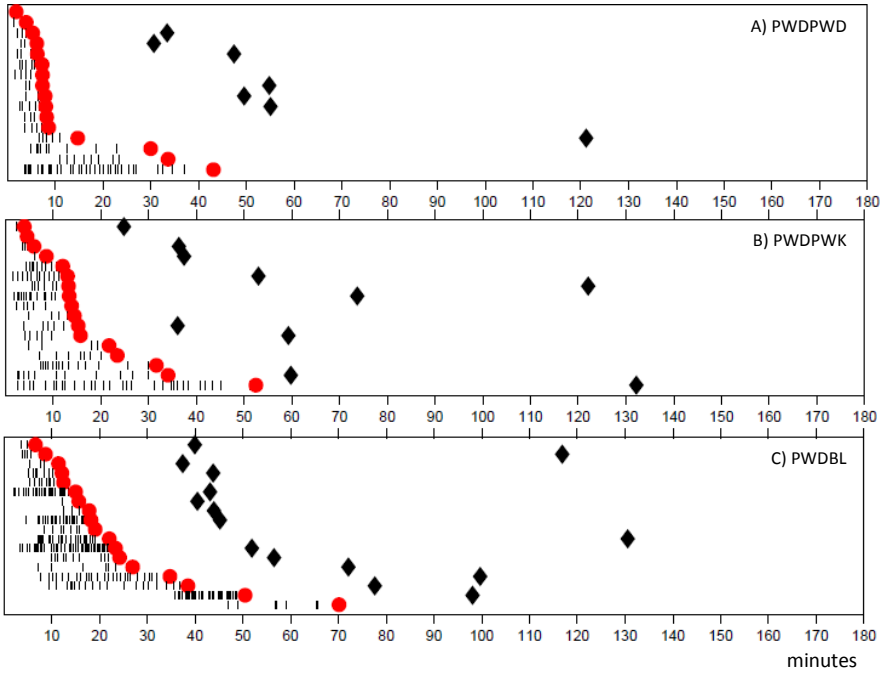

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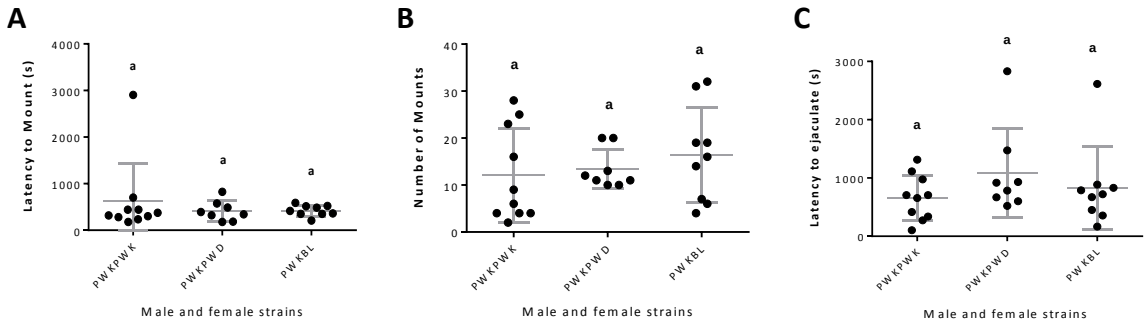
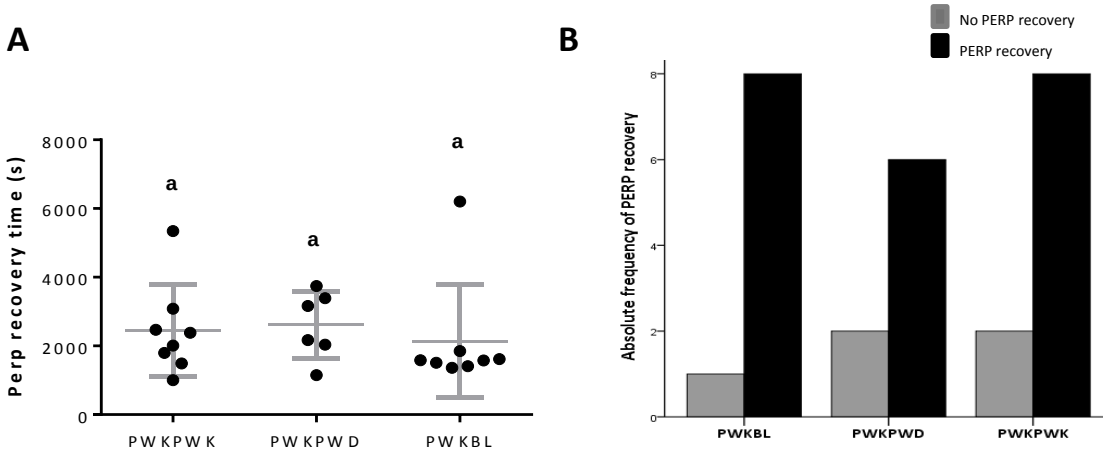
Suplementary data

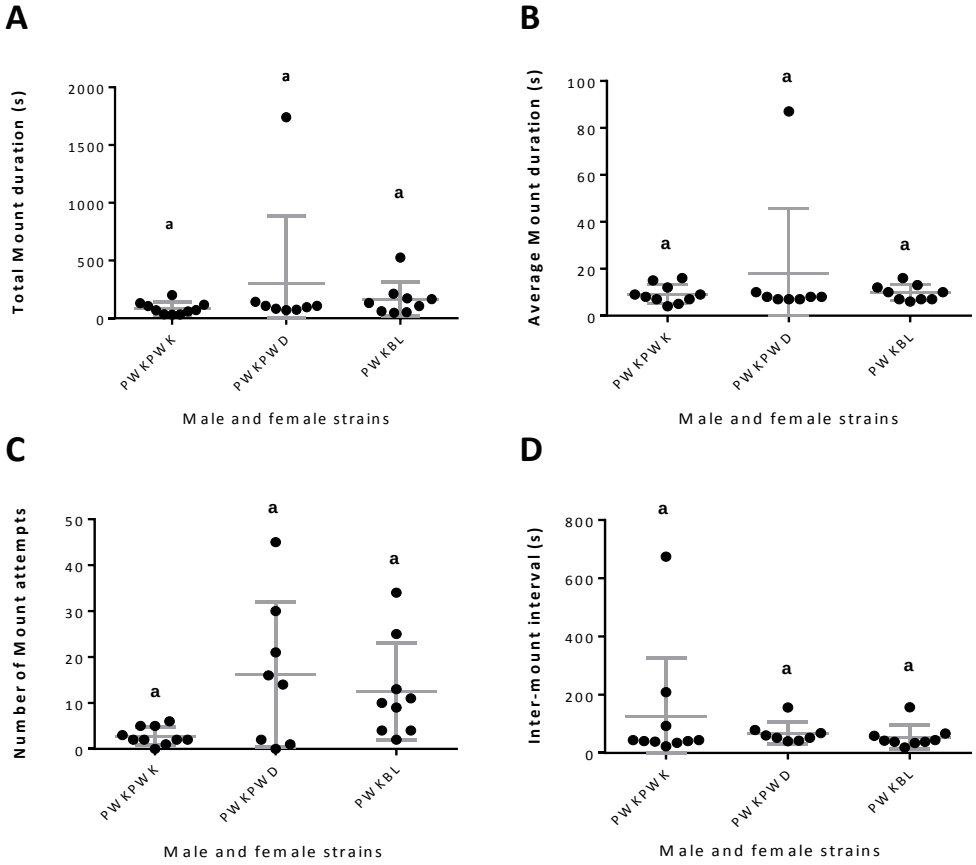
Supplementary figure 1. *Mus musculus musculus* PWK male sexual behaviour with different female strains. Raster plots are aligned to the insertion of the female in the behavioural paradigm and depict mount events (black ticks), ejaculation (red circles) and the first mount event after ejaculation (black diamonds) for A) PWK male x PWK female, N=10; B) PWK male x PWD female, N=8; C) PWK male x Bl female, N=9.



Supplementary figure 2.PWK male sexual behaviour with different female subspecies. A) Latency to mount in seconds (X ± SD PWKPWK = 618.4 ± 816.5, N=10; X ± SD PWKPWD = 413.4 ± 214.9, N=8; X ± SD PWKBL = 422.2 ± 117.5, N=9). B) Number of mounts (X ± SD PWKPWK = 12.10 ± 9.99, N=10; X ± SD PWKPWD = 13.38 ± 4.21, N=8; X ± SD PWKBL = 16.44 ± 10.16, N=9). C) Latency to ejaculate in seconds (X ± SD PWKPWK = 660.4 ± 388.5, N=10; X ± SD PWKPWD = 1091 ± 761.9, N=8; X ± SD PWKBL = 831.6 ± 709.8, N=9). One way ANOVA Kruskal-Wallis test, $p>0.05$; Equal letter (a) indicate a $p>0.05$ obtained by Bonferroni-Dunn's multiple comparisons test.



Supplementary figure 3. A) Post ejaculatory refractory period in seconds for PWK male mice to start mount females of different subspecies (X ± SD PWKPWK = 2447 ± 1332, N=8; X ± SD PWKPWD = 2608 ± 985.0, N=6; X ± SD PWKBL = 2138 ± 1648, N=8). One way ANOVA Kruskal-Wallis test, p>0.05; Equal letter (a) indicate a p>0.05 obtained by Bonferroni-Dunn's multiple comparisons test. B) Frequency of PWK males that restarted to mount after an ejaculation with females of different subspecies. Pearson Qui-square p<0,05 (association between strains and probability to recover from PERP).



Supplementary figure 4.PWK male sexual traits with different female subspecies. A) Total mount duration in seconds (X ± SD PWKPWK = 84.90 ± 54.32, N=10; X ± SD PWKPWD = 302.3 ± 581.4, N=8; X ± SD PWKBL = 164.1 ± 147.3, N=9). B) Average mount duration in seconds (X ± SD PWKPWK = 9.2 ± 3.99, N=10; X ± SD PWKPWD = 17.75 ± 28.00, N=8; X ± SD PWKBL = 9.78 ± 3.38, N=9). C) Number of mount attempts (X ± SD PWKPWK = 12.10 ± 9.99, N=10; X ± SD PWKPWD = 13.38 ± 4.21, N=8; X ± SD PWKBL = 16.44 ± 10.16, N=9). D) Inter-mount interval in seconds (X ± SD PWKPWK = 124.3 ± 200.7, N=10; X ± SD PWKPWD = 68.63 ± 37.60, N=8; X ± SD PWKBL = 55.33 ± 40.57, N=9). One way ANOVA Kruskal-Wallis test, $p>0.05$; Equal letter (a) indicate a $p>0.05$ obtained by Bonferroni-Dunn's multiple comparisons test.